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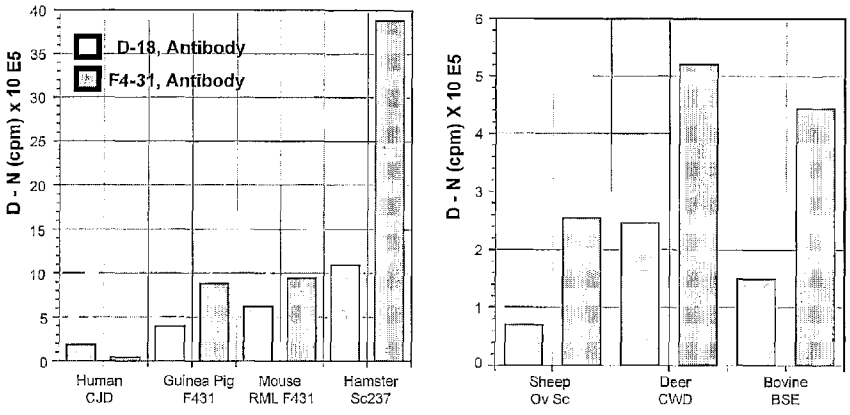
*[Continued on next page]*

(54) Title: ANTIBODIES SPECIFIC FOR HUMAN AND BOVINE PrP

Figure 10 Measurement of infectious prions using a CDI incorporating antibody D-18 versus monoclonal antibody F4-31 as the capture antibody. Samples analyzed were Human, CJD; Guinea Pig, F431; Mouse, RML431; Hamster, Sc237, Sheep, Ov, Deer, CWD and Bovine, BSE infectious brain homogenates.




(57) Abstract: The present invention provides antibodies that specifically bind with a high degree of binding affinity to a native bovine $PrP^{C}$ and/or a denatured bovine $PrP^{Sc}$, but not to a native bovine $PrP^{Sc}$. The antibodies are useful as capture reagents for solid substrate format immunoassays. Such antibodies can be used in an assay to determine if a sample is infected with infectious prions, i.e. $PrP^{Sc}$.

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# ANTIBODIES SPECIFIC FOR HUMAN AND BOVINE PrP

## GOVERNMENT RIGHTS

[0001] This invention was made with government support a Specific Cooperative Agreement No. 58-5325-3-246 with the USDA Agricultural Research Service. The United States Government may have certain rights in this invention.

## ATCC DEPOSIT INFORMATION

[0002] The cell lines F20-29, F20-49, F20-80, F20-89a, F20-108a, F20-130a and F4-31 have been deposited with the American Type Culture Collection and designated Patent Deposit No. PTA-6607. The deposited cell line F4-30 was assigned ATC No. PTA-6838 and the cell line F4-7 was assigned ATC No. PTA-6839.

## FIELD OF THE INVENTION

[0003] This invention relates to antibodies, methods for obtaining antibodies and assays for using such antibodies, including double capture immunoassays. More specifically, the invention relates to antibodies that specifically bind to PrP from humans, bovines, and other mammals.

## BACKGROUND OF THE INVENTION

[0004] Prions are infectious pathogens that cause central nervous system spongiform encephalopathies in humans and animals. Prions are distinct from bacteria, viruses and viroids. The predominant hypothesis at present is that no nucleic acid component is necessary for infectivity of prion protein.

[0005] A major step in the study of prions and the diseases that they cause was the discovery and purification of a protein designated prion protein ("PrP"). $PrP^{C}$ is encoded by a single-copy host gene and is normally found at the outer surface of neurons. A leading hypothesis is that prion diseases result from conversion of $PrP^{C}$ into a modified form called $PrP^{Sc}$. It appears that $PrP^{Sc}$ is necessary for both the transmission and pathogenesis of the transmissible neurodegenerative diseases of animals and humans. See Prusiner, S.B.(1991), Science *252*:1515-1522. The most common prion diseases of animals are scrapie of sheep and goats, and bovine spongiform encephalopathy (BSE) of cattle. Four prion diseases of humans have been identified: (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Strassler-

Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI) (Gajdusek, D.C., (1977) Science *197*:943-960; Medori et al.(1992), N. Engl. J. Med. *326*:444-449).

**[0006]** BSE is a major socioeconomic problem. In Britain, more than 175,000 cattle, primarily dairy cows, have died of BSE during the past decades. More recently, Canada has suffered from this problem. Recently Canadian officials reported finding a new case of BSE in a cow that was born after feed restrictions intended to prevent the spread of the disease were put in place in 1997. The outbreak has cost the Canadian cattle industry $4 billion and more than 6,000 jobs, and it has caused hardship in many small communities in the prairie provinces. Many of Canada's trading partners closed their markets to Canada's beef after a 2003 case of BSE was discovered. The discovery of a BSE case in a US cow has resulted in closure of some export markets for US beef products. BSE has been reported in cows from numerous countries including the United Kingdom, Japan, France, Germany, Italy, and Switzerland.

**[0007]** The importance of detecting BSE has been heightened by the realization that bovine prions have been transmitted to humans who developed new variant Creutzfeldt-Jakob disease (nvCJD) (G. Chazot et al.(1996), *Lancet 347*:1181; R.G. Will et al. (1996), *Lancet 347*:921-925). The transmission of nvCJD from cattle to humans has been confirmed through *in vivo* testing.

**[0008]** The presence of $PrP^{Sc}$ in tissues of humans or animals is indicative of prion infection. $PrP^{Sc}$ is the invariant component of prion infection and is the only disease-specific diagnostic marker that can be readily detected by immunoassay in the brains of clinically ill animals and humans Meyer et al. (1986), *Proc. Natl. Acad. Sci. USA*, *83*:3693-7; Serban et al. (1990), *Neurology, 40*:110-117; Taraboulos et al. (1992), *Proc. Natl. Acad. Sci. USA. 89*:7620-7624; Grathwohl, K. U. D., M. Horiuchi et al. (1997), Virol. Methods *64*:205-216. Unfortunately, $PrP^{Sc}$ assays are positive only when the prion titer is high, and detection of low levels of $PrP^{Sc}$ has been problematic. It has also proven difficult to measure low levels of $PrP^{Sc}$ in the presence of high levels of $PrP^{C}$.

**[0009]** Given the enormity of the potential effect of BSE on world wide cattle populations and trade, there is a great need for a method of assessing bovine infection with BSE to protect cattle populations and consumers. Given the potential health risk to the human population, more sensitive methods for detection of bovine prions are urgently needed to ensure a safe food supply.

## SUMMARY OF THE INVENTION

**[0010]** The present invention provides antibodies that will specifically bind with a high degree of affinity to human $PrP^{C}$ and to bovine $PrP^{C}$ and/or a denatured human or bovine $PrP^{Sc}$, particularly a denatured human or bovine PrP sequence, and are useful as capture reagents, *e.g.* for use in ELISA type assays. Such antibodies may be broadly cross-reactive with PrP from other mammalian species, e.g. mouse, hamster, human, bovine, ovine, deer, elk, and the like. In one embodiment of the invention, the antibody is specific for an epitope in the region from residues 102-241 of bovine PrP and in the region from residues 90-231 of human PrP.

**[0011]** The antibodies are useful in numerous applications, and particularly for determining prion infection in humans and in bovines. The antibodies are characterized by one or more of the following features: (1) an ability to bind to native $PrP^{C}$ or denatured human or bovine $PrP^{Sc}$ with specificity; and (2) an ability to capture various forms of PrP protein in a solids substrate format. Useful capture antibodies need not have a high degree of species specificity, e.g. may cross-reactive with PrP from multiple mammalian species.

**[0012]** An important object is to provide antibodies that bind to bovine and to human $PrP^{C}$ and that are useful in a capture immunoassay.

**[0013]** Still another object is to provide specific methodology for high throughput screening of monoclonal antibodies for usefulness in capture assays.

**[0014]** Another object of the invention is to provide an assay for the detection of $PrP^{Sc}$ particularly in homogenized human or bovine brain tissue using the antibodies of the invention.

**[0015]** An advantage of the invention is that it provides a fast, cost effective assay for detecting the presence of infectious prions in the form of $PrP^{Sc}$ in a bovine or human sample.

**[0016]** A specific advantage is that the assay can be used as a screen for the presence of prions (i.e., $PrP^{Sc}$) in products such as pharmaceuticals (derived from natural sources) food, cosmetics, blood, or any material which might contain such prions and thereby provide further assurances as to the safety of such products.

**[0017]** Another advantage is that the antibodies can be used with a compound which denatures $PrP^{Sc}$ to the degree needed to expose an epitope which will bind to an antibody of the invention thereby providing for a means of differentiating levels of $PrP^{C}$ and $PrP^{C} + PrP^{Sc}$ in a sample.

**[0018]** An aspect of the invention is to provide a therapeutic antibody which prevents or treats prion disease in bovines and specifically in cows.

[0019] Another aspect of the invention is to provide a means for certifying certain products as being prion free.

[0020] These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the hybridoma, assay method and antibodies as more fully described below.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

[0022] Figure 1 is a schematic of a double capture immunoassay (DCI).

[0023] Figure 2A is a graph of a DCI analysis of sera from individual mice immunized with recBoPrP102-241.

[0024] Figure 2B shows a Western blot analysis of brain homogenate from PrP ablated mouse brain, $PrP^{0/0}$; mouse; Syrian hamster; Human; bovine; recombinant bovine, recBo, sheep; and mule deer. PrP was detected using serum from a mouse immunized with recBoPrP102-241.

[0025] Figure 3A is a graph depicting ELISA activity of Protein G purified F4-31 antibody. Figure 3B shows a silver stained SDS-Page gel analysis of Protein-G purified F4-31 antibody. Markers, lane 1; non-reduced sample, lane 2; reduced sample, lane 3.

[0026] Figure 4 is a graph of a DCI titration analysis of F4-31 monoclonal antibody on Human recPrP90-231 versus Bovine recPrP102-242.

[0027] Figure 5 shows a Western blot analysis of brain homogenate from PrP ablated mouse brain, $PrP^{0/0}$; mouse, Mo; Syrian hamster, SHa; Human, Hu; bovine, Bo; recombinant bovine, recBo, sheep, Ov; and mule deer, MD detected with monoclonal antibody F4-31.

[0028] Figures 6A-6B show antibody binding to synthetic peptides.

[0029] Figure 7 Shows electrophoretic analysis and western blot analysis of reduced (lanes 1, 3, 5) and non-reduced (lanes 2, 4, 6) recBoPrP102-241 stained with silver (lanes 1-2), probed with antibody HumP (lanes 3-4) or probed with monoclonal antibody F4-31 (lanes 5-6).

[0030] Figure 8 is a graph depicting results of an ELISA titration on reduced (DTT treated) and non reduced antigen. In Figure 8A the monoclonal antibodies F4-31, F20-29, F20-80 and F20-130a show loss of binding to the reduced antigen. In Figure 8B binding of the monoclonal antibodies F20-49 and F20-89 is not affected by the reduction of the antigen.

[0031] Figure 9 is a graph depicting the effect of GndHCl treatment of recBoPrP102-241 or recHuPrP90-231, on subsequent antibody binding in a direct binding ELISA. Figure 9A shows the results for monoclonal antibodies F4-31, F20-29, F20-49 and F20-80. Figure 9B shows the results for monoclonal antibodies F20-89, F20-108a and F20-130a. HuM-P is shown as a control.

[0032] Figure 10, panels A and B show detection B of infectious prions using a CDI incorporating antibody D-18 (a previously described antibody) versus monoclonal antibody F4-31 as the capture antibody. In panel A the samples analyzed were Human, CJD; Guinea Pig, F431; Mouse, RML431; and Hamster, Sc237. In panel B the samples were Sheep, Ov, Deer, CWD; and Bovine, BSE infectious brain homogenates.

[0033] Figure 11 is a graph showing the results of determining the capture affinity constant (Ka) of the monoclonal antibody F4-31.

[0034] Figure 12 is a graph of the results of a dissolution curve of BSE infected brain homogenate into normal cow brain homogenate.

## DETAILED DESCRIPTION OF THE INVENTION

[0035] Before description of the present antibodies, assays and methods it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0036] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supercedes any disclosure of an incorporated publication to the extent there is a contradiction.

**[0038]** It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an antibody" includes a plurality of such antibodies and reference to "the hybridoma" includes reference to one or more hybridomas and equivalents thereof known to those skilled in the art, and so forth.

**[0039]** The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

## DEFINITIONS

**[0040]** The terms "PrP protein", "PrP" and the like are used interchangeably herein and shall mean both the infectious particle form $PrP^{Sc}$ known to cause diseases (spongiform encephalopathies) in humans and animals and the non-infectious form $PrP^{C}$ which, under appropriate conditions is converted to the infectious $PrP^{Sc}$ form.

**[0041]** The terms "prion", "prion protein" and "$PrP^{Sc}$ protein" and the like used interchangeably herein to refer to the infectious $PrP^{Sc}$ form of a PrP protein and is a contraction of the words "protein" and "infection" and the particles are comprised largely if not exclusively of $PrP^{Sc}$ molecules encoded by a PrP gene. Prions are distinct from bacteria, viruses and viroids. Known prions include those which infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats as well as bovine spongiform encephalopathies (BSE) or mad cow disease and chronic wasting disease (CWD) of deer and elk. Four prion diseases known to affect humans are (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Strassler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI). As used herein prion includes all forms of prions causing all or any of these diseases or others in any animals used — and in particular in humans and in domesticated farm animals.

[0042] The term "PrP gene" is used herein to describe genetic material that encodes $PrP^C$ proteins, including proteins having polymorphisms and mutations such as those listed herein under the subheading "Pathogenic Mutations and Polymorphisms." The term "PrP gene" refers generally to any gene of any species which encodes any form of a prion protein. Some commonly known PrP sequences are described in Gabriel et al. (1992), Proc. Natl. Acad. Sci. USA *89*:9097-9101, which is incorporated herein by reference to disclose and describe such sequences. The PrP gene can be from any animal including the "host" and "test" animals described herein and any and all polymorphisms and mutations thereof, it being recognized that the terms include other such PrP genes that are yet to be discovered. The protein expressed by such a gene can assume either a $PrP^C$ (non-disease) or $PrP^{Sc}$ (disease) form.

[0043] The terms "standardized prion preparation", "prion preparation", "preparation" and the like are used interchangeably herein to describe a composition containing prions ($PrP^{Sc}$) which composition is obtained from brain tissue of mammals which contain substantially the same genetic material as relates to prions, *e.g.*, brain tissue from a set of mammals which exhibit signs of prion disease which mammals (1) include a transgene as described herein; (2) have an ablated endogenous prion protein gene; (3) have a high copy number of prion protein gene from a genetically diverse species; or (4) are hybrids with an ablated endogenous prion protein gene and a prion protein gene from a genetically diverse species. The mammals from which standardized prion preparations are obtained exhibit clinical signs of CNS dysfunction as a result of inoculation with prions and/or due to developing the disease due to their genetically modified make up, *e.g.*, high copy number of prion protein genes.

[0044] The term "genetic material related to prions" is intended to cover any genetic material which effects the ability of an animal to become infected with prions. Thus, the term encompasses any "PrP gene", "artificial PrP gene", "chimeric PrP gene" or "ablated PrP gene" which terms are defined herein as well as modification of such which effect the ability of an animal to become infected with prions. Standardized prion preparations are produced using animals which all have substantially the same genetic material related to prions so that all of the animals will become infected with the same type of prions and will exhibit signs of infection at about the same time.

[0045] The terms "host animal" and "host mammal" are used to describe animals which may have their genome genetically and artificially manipulated so as to include genetic material which is not naturally present within the animal. For example, host animals include mice, hamsters and rats which have their PrP gene ablated, i.e., rendered inoperative. The host is

inoculated with prion proteins to generate antibodies. Other host animals can have a natural (PrP) gene or one which is altered by the insertion of an artificial gene or by the insertion of a native PrP gene of a genetically diverse test animal.

**[0046]** The terms "test animal" and "test mammal" are used to describe the animal which is genetically diverse from the host animal in terms of differences between the PrP gene of the host animal and the PrP gene of the test animal. The test animal may be any animal for which one wishes to run an assay test to determine whether a given sample contains prions with the ability to infect test animal. For example, the test animal may be any bovine or mammal infected with a variant bovine prion, including human, cow, sheep, pig, horse, cat, dog or chicken, and one may wish to determine whether a particular sample includes prions which would normally infect only the test animal.

**[0047]** The terms "genetically diverse animal" and "genetically diverse mammal" are used herein to describe an animal which includes a native PrP codon sequence of the host animal differing from the genetically diverse test animal by 17 or more codons, preferably 20 or more codons, and most preferably 28-40 codons. Thus, a mouse PrP gene is genetically diverse with respect to the PrP gene of a cow or sheep, but is not genetically diverse with respect to the PrP gene of a hamster.

**[0048]** The terms "ablated PrP protein gene", "disrupted PrP gene", and the like are used interchangeably herein to mean an endogenous PrP gene which has been altered (*e.g.*, added and/or removed nucleotides) in a manner so as to render the gene inoperative. Examples of non-functional PrP genes and methods of making such are disclosed in Büeler, H., et al. (1992), *Nature* 356, 577-582 and Weissman (WO 93/10227). The methodology for ablating a gene is taught in Capecchi (1987), *Cell* 51:503-512, all of which are incorporated herein by reference. Preferably both alleles of the genes are disrupted.

**[0049]** The terms "hybrid animal", "transgenic hybrid animal" and the like are used interchangeably herein to mean an animal obtained from the cross-breeding of a first animal having an ablated endogenous prion protein gene with a second animal which includes either (1) a chimeric gene or artificial PrP gene or (2) a PrP gene from a genetically diverse animal. For example a hybrid mouse is obtained by cross-breeding a mouse with an ablated mouse gene with a mouse containing (1) bovine or other bovine PrP genes (which may be present in high copy numbers) or (2) chimeric mouse/bovine PrP genes. The term hybrid includes any offspring of a hybrid including inbred offspring of two hybrids provided the resulting offspring is susceptible to infection with prions with normal infect only a genetically diverse species. A

hybrid animal can be inoculated with prions and serve as a source of cells for the creation of hybridomas to make monoclonal antibodies of the invention.

[0050] The terms "susceptible to infection" and "susceptible to infection by prions" and the like are used interchangeably herein to describe a transgenic or hybrid test animal which develops a disease if inoculated with prions which would normally only infect a genetically diverse test animal. The terms are used to describe a transgenic or hybrid animal such as a transgenic mouse Tg(MBo2M) which, without the chimeric PrP gene, would not become infected with a bovine prion but with the chimeric gene is susceptible to infection with bovine prions.

[0051] The term "ungulate" as used herein refers to any hoofed mammal. This includes, but is not limited to, cows, deer, elk, sheep and goats. For purposes of the invention a preferred ungulate is a bovine (cow).

[0052] The term "antibody" refers to an immunoglobulin protein that is capable of binding an antigen. The archetypal antibody molecule is the immunoglobulin, and all types of immunoglobulins, IgG, IgM, IgA, IgE, IgD, etc., from all sources, e.g. human, rodent, rabbit, cow, sheep, pig, dog, other mammal, chicken, other avians, *etc.*, are considered to be "antibodies." Antibody as used herein is meant to include the entire antibody as well as any antibody fragments (e.g. F(ab', Fab, Fv) capable of binding the epitope, antigen or antigenic fragment of interest. The term "antibody" encompasses all types of antibodies, e.g. polyclonal, monoclonal, *etc.*, however for most purposes of the invention, reference is made to monoclonal antibodies. Monoclonal antibodies are preferred for many uses because they can be reproduced by cell culture or recombinantly, and can be modified to reduce their antigenicity. Particularly preferred antibodies of the invention are antibodies that have a relatively high degree of affinity for the target antigen.

[0053] "Purified antibody" refers to that which is sufficiently free of other proteins, carbohydrates, and lipids with which it is naturally associated. Such an antibody "preferentially binds" to its cognate antigen with high specificity, and does not substantially recognize or bind to other antigenically unrelated molecules. A purified antibody of the invention may be immunoreactive with, and immunospecific for, a specific species; or may be cross-reactive with PrP proteins of different mammalian species.

[0054] By "antigenic fragment" of a PrP protein is meant a portion of such a protein which is capable of binding an antibody of the invention.

[0055] The level of affinity of antibody binding that is considered to be "specific" will be determined in part by the class of antibody, e.g. antigen specific antibodies of the IgM class may have a lower affinity than antibodies of, for example, the IgG classes. As used herein, in order to consider an antibody interaction to be "specific", the affinity will be at least about $10^{-7}$ M, usually about $10^{-8}$ to $^{-9}$ M, and may be up to $10^{-11}$ or higher for the epitope of interest. It will be understood by those of skill in the art that the term "specificity" refers to such a high affinity binding, and is not intended to mean that the antibody cannot bind to other molecules as well. One may find cross-reactivity with different epitopes, due, e.g. to a relatedness of antigen sequence or structure, or to the structure of the antibody binding pocket itself. Antibodies demonstrating such cross-reactivity are still considered specific for the purposes of the present invention.

[0056] By "detectably labeled antibody", "detectably labeled anti-PrP" or "detectably labeled anti-PrP fragment" is meant an antibody (or antibody fragment which retains binding specificity), having an attached detectable label. The detectable label is normally attached by chemical conjugation, but where the label is a polypeptide, it could alternatively be attached by genetic engineering techniques. Methods for production of detectably labeled proteins are well known in the art. Detectable labels may be selected from a variety of such labels known in the art, but normally are radioisotopes, fluorophores, paramagnetic labels, enzymes (*e.g.*, horseradish peroxidase), or other moieties or compounds which either emit a detectable signal (*e.g.*, radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate. Various detectable label/substrate pairs (*e.g.*, horseradish peroxidase/diaminobenzidine, avidin/streptavidin, luciferase/luciferin)), methods for labeling antibodies, and methods for using labeled antibodies are well known in the art (see, for example, Harlow and Lane, eds. (*Antibodies: A Laboratory Manual* (1988) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)).

[0057] The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly humans, and bovine, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it;

(b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease.

## General Aspects of the Invention

**[0058]** The present invention provides an antibody that specifically binds to a human or bovine $PrP^C$, usually a denatured human or bovine PrP wherein all PrP has been treated in a limited manner in order to expose epitopes that bind an antibody of the invention. Preferably the antibody is useful as a capture reagent, and may be cross-reactive with other mammalian PrP proteins. In one aspect, an assay is provided for screening antibodies for use in a capture assay, depicted in **Figure 1**.

**[0059]** This newly developed rapid double capture immunoassay (DCI) allows for early (at the first step in the screening process) identification of monoclonal antibodies that are useful as capture reagents. Microtiter wells are coated with an antibody (referred to as antibody 1) that binds to the constant (Fc) region of the antibody expressed by the newly fused hybridoma cells, *e.g.* a goat anti-mouse IgG (Fc specific); or a goat anti-rat IgG; *etc.*

**[0060]** Reactive sites on the surface of the plastic well are then blocked from further reactivity (binding) with proteins used in subsequent steps by addition of blocking buffer containing 5% nonfat dry milk. Hybridoma supernatants comprising a candidate monoclonal antibody (antibody 2) (e.g., mouse monoclonal antibodies produced by hybridomas following cell fusion) are then added to the microtiter wells and the plate incubated for a period of time sufficient to allow any antibody 2 (e.g., mouse antibodies present in the hybridoma supernatants) to specifically bind via interaction with the combining site of the immobilized antibody 1 (e.g., the goat anti-mouse antibody) . Using a mouse hybridoma as example, the plate is coated with goat-anti-mouse antibody and when hybridoma supernatants are screened to determine if they contain mouse monoclonal antibodies of interest, the hybridoma supernatant is added into the microtiter well, any mouse monoclonal antibodies present in the supernatant will specifically bind to the the anti-mouse antibody (antibody 1) by reaction of the constant region of the mouse monoclonal antibody with the combining site of the immobilized goat-anti-mouse antibody. The mouse monoclonal antibodies are now immobilized on the surface of the microtiter well and are oriented so that their combining sites (in this example, that portion that binds prion protein) is available to bind antigen. The supernatant is removed, the plate washed and the combining site of antibody 1 not occupied with antibodies in the hybridoma supernatants are blocked by allowing an irrelevant antibody molecule (in this

example, a nonspecific mouse immunoglobulin) to bind the nonoccupied combining sites of antibody 1. This second blocking step reduces background fluorescence resulting from an apparent binding of the unoccupied combining sites of antibody 1 with subsequently added reagents. The plates are decanted, the cognate antigen dissolved in blocking buffer is added, and the plate incubated for a period of time sufficient for the antigen to bind to the trapped monoclonal antibodies (antibody 2). The plates are then washed, and Europium conjugated anti-prion antibody is added. Finally, the plates are washed, enhancement solution is added, and fluorescence (time-resolved) intensity measured. A positive signal is observed only if the Europium conjugated antibody binds to prion protein and prion protein will be pesent only if the hybridoma supernatants being evaluated contained anti-prion antibodies that reacted with antibody 1.

**[0061]** More specifically, the methods of the invention provide for the development of antibodies that are able to recognize epitopes that are present on both $PrP^{C}$ and treated $PrP^{Sc}$ i.e. denatured prion proteins.

**[0062]** In one embodiment, the antibody is produced by one of the hybridoma cell lines F4-31, F4-30 or F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a, or F20-130a or an antibody that specifically binds to the epitope specifically recognized by the antibody produced by one of the hybridoma cell lines F4-31, F4-30 or F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a, or F20-130a

**[0063]** Preferably, the antibodies specifically bind to a denatured human or bovine $PrP^{Sc}$ protein *in situ* with an affinity of $10^{7}$ moles/liter or more, preferable $10^{8}$ moles/liter or more of a single species. Antibodies of the invention may have an affinity for multiple species, *e.g.*, humans, all types of ungulates including cows, , sheep, goats, etc. or may be specific to a single species, *e.g.,* cow or human. Antibodies may be isolated, using the protocols of the present invention, with the ability to bind to all proteins coded by the different mutations and/or polymorphisms of the human and bovine PrP protein gene. Alternatively, a battery of antibodies (2 or more different antibodies) can be provided wherein each antibody of the battery specifically binds to a protein encoded by a different mutation or polymorphism of a human or a bovine PrP gene. Thus, the antibody can be bound to a support surface and used to assay a sample *in vitro* for the presence of a particular allele of human or of bovine $PrP^{C}$.

**[0064]** The antibodies of the present invention are especially useful to detect prions utilizing *in vitro* methods, in which the presence of $PrP^{Sc}$ in tissues of humans or animals (e.g, homogenated bovine brain) indicates prion infection. A conformation-dependent

immunoassay (CDI) such as disclosed in U.S. Patent 5,891,641 offers a rapid, specific, and highly sensitive method for the detection of bovine $PrP^{Sc}$ using the antibodies of the invention. The assay, as the name indicates, is conformation-sensitive and can detect relatively low levels of $PrP^{Sc}$ in brain homogenates in which $PrP^{C}$ is present in a 100-fold excess relative to $PrP^{Sc}$.

**[0065]** Rapid application of CDI for early detection of BSE prions in different tissues of humans or cows can be complicated by the lack of high-affinity antibody reacting with denatured human or bovine PrP. Conformational sensitivity of CDI is crucial for specificity of the assay and the ability to distinguish $PrP^{Sc}$ from $PrP^{C}$. The methods of the invention provide the rational development and specific selection of high-affinity anti-bovine $PrP^{C}$ and anti-human $PrP^{C}$ antibodies that can be used in, among other things, conformation-dependent immunoassays (CDI), for example, in assays for wild type and *de novo* bovine, human, sheep, and deer prions. A CDI assay is described in U.S. Patents 5,891,641 issued April 6, 1999, 6,617,119 and 6,620,629 are incorporated herein by reference in their entirety. The selection of antibodies and resultant assays can be performed directly in samples or indirectly in the brains of animals inoculated with a sample containing prions.

**[0066]** To produce antibodies of the invention it is preferable to begin with inoculating a host mammal with an inoculum from the desired human or bovine $PrP^{C}$. The host mammal may be any mammal and is preferably a host mammal of the type defined herein such as a mouse, rat, rabbit, guinea pig or hamster, and is most preferably a mouse. The host animal is inoculated either with PrP proteins that are endogenous to humans or to a bovine species. For example a mouse is inoculated with a bovine $PrP^{C}$ peptide which may be treated to expose epitopes. Using a normal host mammal in this manner it is possible to elicit the generation of some antibodies, however, since the host animal includes a PrP protein gene and if inoculated with $PrP^{C}$ from a genetically diverse species, the antibodies will, if at all, only be generated for epitopes which differ between epitopes of the PrP protein of the host animal and epitopes of the $PrP^{C}$ from the genetically diverse species. This substantially limits the amount of antibodies that might be generated and decreases the ability to find an antibody that selectively binds to a human or bovine $PrP^{C}$.

**[0067]** Therefore it is preferable that antibodies be generated in animals that have an ablated PrP protein gene, i.e., a null PrP gene abbreviated as $Prnp^{0/0}$. This allows antibodies to be generated against areas of bovine $PrP^{C}$ that are conserved between the host animal and the bovine PrP genes. Accordingly, the invention is also described in connection with the use of such "null" mammals and more specifically described in connection with "null mice."

[0068] A null mouse can be created by inserting a segment of DNA into a normal mouse PrP gene and/or removing a portion of the gene to provide a disrupted PrP gene. The disrupted gene is injected into a mouse embryo and replaces the endogenous PrP gene via homologous recombination.

[0069] The null mouse is injected with either human or bovine PrP peptides to stimulate the formation of antibodies. Injections of adjuvants can be used in conjunction with the peptides to maximize the generation of antibodies. The mouse is then sacrificed and bone marrow and spleen cells are removed.

[0070] Hybridomas are generated by conventional methods. Immunization may be performed using a standard protocol, by injecting the null animal with an antigenic composition, see, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. The protein, peptide, peptide-conjugate, etc. is injected into a animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the antigen may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

[0071] Hybridomas may be formed by isolating the stimulated immune cells, such as those from the spleen of the inoculated animal. These cells are then fused to immortalized cells, such as myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The immortal cell line utilized is preferably selected to be deficient in enzymes necessary for the utilization of certain nutrients. Many such cell lines (such as myelomas) are known to those skilled in the art, and include, for example: thymidine kinase (TK) or hypoxanthine-guanine phosphoriboxyl transferase (HGPRT) deficient lines. These deficiencies allow selection for fused cells according to their ability to grow on, for example, hypoxanthine-aminopterin-thymidine medium (HAT). The fusion partner is preferably altered in a manner such that they have reduced expression or no expression of the PrP protein (to which the antibody binds).

[0072] In the generation of the hybridoma, preferably, the immortal fusion partners utilized are derived from a line that does not secrete immunoglobulin. The resulting fused cells, or hybridomas, are cultured under conditions that allow for the survival of fused, but not unfused, cells and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, expanded, and grown so as to

produce large quantities of antibody, see Kohler and Milstein, 1975 Nature 256:495 (the disclosures of which are hereby incorporated by reference).

[0073] Large quantities of monoclonal antibodies from the secreting hybridomas can be produced by injecting the clones into the peritoneal cavity of mice and harvesting the ascites fluid therefrom. The mice, preferably primed with pristane, or some other tumor-promoter, and immunosuppressed chemically or by irradiation, may be any of various suitable strains known to those in the art. The ascites fluid is harvested from the mice and the monoclonal antibody purified therefrom, for example, by CM Sepharose column or other chromatographic means. Alternatively, the hybridomas may be cultured *in vitro* or as suspension cultures. Batch, continuous culture, or other suitable culture processes may be utilized. Monoclonal antibodies are then recovered from the culture medium or supernatant.

[0074] Chimeric antibodies may be made by recombinant means by combining the murine variable light and heavy chain regions (VK and VH), obtained from a murine (or other animal-derived) hybridoma clone, with the human constant light and heavy chain regions, in order to produce an antibody with predominantly human domains. The production of such chimeric antibodies is well known in the art, and may be achieved by standard means (as described, e.g., in U.S. Patent No. 5,624,659, incorporated fully herein by reference). Humanized antibodies are engineered to contain even more human-like immunoglobulin domains, and incorporate only the complementarity-determining regions of the animal-derived antibody. This is accomplished by carefully examining the sequence of the hyper-variable loops of the variable regions of the monoclonal antibody, and fitting them to the structure of the human antibody chains. Although facially complex, the process is straightforward in practice. See, e.g., U.S. Patent No. 6,187,287, incorporated fully herein by reference.

[0075] In addition to entire immunoglobulins (or their recombinant counterparts), immunoglobulin fragments comprising the epitope binding site (e.g., Fab', $F(ab')_2$, or other fragments) are useful as antibody moieties in the present invention. Such antibody fragments may be generated from whole immunoglobulins by ficin, pepsin, papain, or other protease cleavage. "Fragment," or minimal immunoglobulins may be designed utilizing recombinant immunoglobulin techniques. For example, "Fv" immunoglobulins for use in the present invention may be produced by linking a variable light chain region to a variable heavy chain region via a peptide linker (e.g., poly-glycine or another sequence which does not form an alpha helix or beta sheet motif).

[0076] Fv fragments are heterodimers of the variable heavy chain domain ($V_H$) and the variable light chain domain ($V_L$). The heterodimers of heavy and light chain domains that occur in whole IgG, for example, are connected by a disulfide bond. Recombinant Fvs in which $V_H$ and $V_L$ are connected by a peptide linker are typically stable, see, for example, Huston et al., Proc. Natl. Acad, Sci. USA 85:5879-5883 (1988) and Bird et al., Science 242:423-426 (1988), both fully incorporated herein, by reference. Improved Fv's have been also been made which comprise stabilizing disulfide bonds between the $V_H$ and $V_L$ regions, as described in U.S. Patent No. 6,147,203, incorporated fully herein by reference.

[0077] In addition, derivatized immunoglobulins with added chemical linkers, detectable moieties, such as fluorescent dyes, enzymes, substrates, chemiluminescent moieties and the like, or specific binding moieties, such as streptavidin, avidin, biotin, or histidine molecules to facilitate purification, and the like may be utilized for various purposes.

## Specifics of a PrP Gene and PrP Proteins

[0078] The genetic material which makes up the PrP gene is known for a number of different species of animals (see Gabriel et al. (1992), *Proc. Natl. Acad. Sci. USA* *89*:9097-9101 and U.S. Patents 5,763,740 and 5,792,901). Further, there is considerable homology between the PrP genes in different mammals. Although there is considerable genetic homology with respect to PrP genes, the differences are significant in some instances. More specifically, due to small differences in the protein encoded by the PrP gene of different mammals, a prion which will infect one mammal (*e.g.* a human) will not normally infect a different mammal (*e.g.* a mouse). Due to this "species barrier", it is not generally possible to use normal animals, (i.e., animal which have not had their genetic material related to PrP proteins manipulated) such as mice to determine whether a particular sample contains prions which would normally infect a different species of animal such as a human. The present invention provides methods for using modified, transgenic animals having bovine PrP genes or a chimeric bovine PrP gene to detect prions in samples from bovines. The antibodies of the present invention provide the means by which these bovine prions can be detected in assays.

[0079] The major component of purified infectious prions, designated PrP 27-30, is the proteinase K resistant core of a larger native protein $PrP^{Sc}$ which is the disease causing form of the ubiquitous cellular protein $PrP^{C}$. $PrP^{Sc}$ is found only in scrapie infected cells, whereas $PrP^{C}$ is present in both infected and uninfected cells implicating $PrP^{Sc}$ as the major, if not the sole, component of infectious prion particles. Since both $PrP^{C}$ and $PrP^{Sc}$ are encoded by the same

single copy gene, great effort has been directed toward unraveling the mechanism by which $PrP^{Sc}$ is derived from $PrP^{C}$. Central to this goal has been the characterization of physical and chemical differences between these two molecules. Properties distinguishing $PrP^{Sc}$ from $PrP^{C}$ include low solubility (Meyer et al.(1986), *Proc. Natl. Acad. Sci. USA* *83*:3693-7), poor antigenicity (Kascsak et al.(1987), "Mouse Polyclonal and Monoclonal Antibody to Scrapie-Associated Fibril Proteins." *J. Virol.* *61*(12):3688-3693; Serban et al.(1990), *Neurology* *40*:110-117) protease resistance (Oesch et al.(1985), *Cell* *40*:735-746) and polymerization of PrP 27-30 into rod-shaped aggregates which are very similar, on the ultrastructural and histochemical levels, to the PrP amyloid plaques seen in scrapie diseased brains (Prusiner, et al (1983) *Cell*). By using proteinase K it is possible to denature $PrP^{C}$ but not $PrP^{Sc}$. It has been proposed that $PrP^{C}$ and $PrP^{Sc}$ are in fact conformational isomers of the same molecule.

**[0080]** Conformational description of PrP using conventional techniques has been hindered by problems of solubility and the difficulty in producing sufficient quantities of pure protein. However, $PrP^{C}$ and $PrP^{Sc}$ are conformationally distinct. Theoretical calculations based upon the amino acid sequences of PrP proteins from several species have predicted four putative helical motifs in the molecule. Experimental spectroscopic data would indicate that in $PrP^{C}$ these regions adopt α-helical arrangements, with virtually no beta-sheet (Pan, K.M. et al (1993) *PNAS* *90*:10962:6). In dramatic contrast, in the same study it was found that $PrP^{Sc}$ and PrP 27-30 possess significant beta-sheet content, which is typical of amyloid proteins. Moreover, studies with extended synthetic peptides, corresponding to PrP amino acid residues 90-145, have demonstrated that these truncated molecules may be converted to either alph-helical or beta-sheet structures by altering their solution conditions. The transition of $PrP^{C}$ to $PrP^{Sc}$ requires the adoption of beta-sheet structure by regions that were previously alpha-helical.

**[0081]** It is not entirely clear as to why antibodies of the type described in the above cited publications will bind to $PrP^{C}$ but not to $PrP^{Sc}$. Without being bound to any particular theory it is suggested that such may take place because epitopes which are exposed when the protein is in the $PrP^{C}$ conformation are unexposed or partially hidden in the $PrP^{Sc}$ configuration -- where the protein is relatively insoluble and more compactly folded together. For purposes of the invention an indication that no binding occurs means that the equilibrium or affinity constant $K_a$ is $10^6$ l/mole or less. Further, binding will be recognized as existing when the $K_a$ is at $10^7$ l/mole or greater preferably $10^8$ l/mole or greater. The binding affinity of $10^7$ l/mole or more may be due to (1) a single monoclonal antibody (i.e., large numbers of one kind of antibodies) (2) a plurality of different monoclonal antibodies (*e.g.*, large numbers of each of five different

monoclonal antibodies) or (3) large numbers of polyclonal antibodies. It is also possible to use combinations of or all of (1)-(3).

## The Conformation-Dependent Assay (CDI)

[0082] The Conformation-Dependent Assay; or "CDI" allows the direct measurement of the amount of $PrP^{Sc}$ in brain homogenates without prior digestion with proteinase K to eliminate $PrP^{C}$. The assay is conformation-sensitive and can detect relatively low levels of $PrP^{Sc}$ in brain homogenates in which $PrP^{C}$ is present in a 100-fold excess. By selective precipitation of $PrP^{Sc}$ prior to differential immunoassay, $PrP^{Sc}$ can be measured in the presence of a 3,000-fold excess of $PrP^{C}$. Currently, the assay can quantify less than 1 ng/ml of $PrP^{Sc}$ in brain homogenate with a dynamic range of 5 orders of magnitude (Safar, J., H. Wille et al. (1998), Nat. Med, 4(10):1157-1165). Since the prion titer in brain homogenates of clinically ill CJD patients is equal to or lower than $10^6$ ID50 units/ml of 5% brain homogenate (unpublished data), the differential immunoassay can detect prion titers as low as 1 ID50 unit/ml.

[0083] The CDI allows one to distinguish multiple strains of prions by plotting the ratio of denatured/native PrP as a function of $PrP^{Sc}$ concentration before and after limited proteinase K digestion. In contrast, only one strain (DY) (Bessen, R. A. and R. F. Marsh (1994), J. Virol. 68:7859-7868) can be distinguished from the other seven strains by Western blotting after limited proteolysis. Moreover, their relativity increased protease sensitivity of $PrP^{Sc}$ in DY prions can lead to an underestimation of its level by immunoblotting (Scott, M. R., D. Groth, et al. (1997), J. Virol. 71:9032-9044).

[0084] Specifically, the antibodies to bovine residues 102-130 (epitope I) allow the CDI to detect prions in cows, deer, elk, sheep and other bovines. Antibodies to human residues 90-120 (epitope 1) allow the CDI to detect prions in humans. The high-affinity antibody reacting within epitope I of the denatured bovine PrP allow the CDI assay to detect, for example, the presence of human or bovine prions in a test sample. This epitope is critical not only for absolute, but also for conformational sensitivity of CDI. Conformational sensitivity of CDI is crucial for specificity of the assay and the ability to distinguish $PrP^{Sc}$ from $PrP^{C}$.

## Pathogenic mutations and polymorphisms

[0085] There are a number of known pathogenic mutations in the human PrP gene. Further, there are known polymorphisms in the human, sheep and bovine PrP genes. The antibodies of the present invention may be geared to recognize specific alleles of the PrP gene.

Alternatively polymorphisms or mutations known to be pathogenic in one species (*e.g. human)* can be added to a peptide from a bovine PrP. The following is a list of such mutations and polymorphisms:

| Pathogenic bovine mutations | Human Polymorphisms | Sheep Polymorphisms | Bovine Polymorphisms |
|---|---|---|---|
| 2 octarepeat insert | Codon 129 Met/Val | Codon 171 Arg/Glu | 5 or 6 octarepeats |
| 4 octarepeat insert | Codon 219 Glu/Lys | Codon 136 Ala/Val | |
| 5 octarepeat insert | | | |
| 6 octarepeat insert | | | |
| 7 octarepeat insert | | | |
| 8 octarepeat insert | | | |
| 9 octarepeat insert | | | |
| Codon 102 Pro-Leu | | | |
| Codon 105 Pro-Leu | | | |
| Codon 117 Ala-Val | | | |
| Codon 145 Stop | | | |
| Codon 178 Asp-Asn | | | |
| Codon 180 Val-Ile | | | |
| Codon 198 Phe-Ser | | | |
| Codon 200 Glu-Lys | | | |
| Codon 210 Val-Ile | | | |
| Codon 217 Asn-Arg | | | |
| Codon 232 Met-Ala | | | |

**[0086]** The DNA sequence of the human, sheep, and cow PrP genes have been determined allowing, in each case, the prediction of the complete amino acid sequence of their respective PrP proteins. The normal amino acid sequence which occurs in the vast majority of individuals is referred to as the wild-type PrP sequence. This wild-type sequence is subject to certain characteristic polymorphic variations. In the case of sheep PrP the gene displays two amino acid polymorphisms at residues 171 and 136, while bovine PrP has either five or six repeats of an eight amino acid motif sequence in the amino terminal region of the mature prion

protein. While none of these polymorphisms are of themselves pathogenic, they appear to influence prion diseases. Distinct from these normal variations of the wild-type PrP proteins, certain mutations of the human PrP gene which alter either specific amino acid residues of PrP or the number of octarepeats have been identified which segregate with inherited human prion diseases.

[0087] In order to provide further meaning to the above chart demonstrating the mutations and polymorphisms, one can refer to the published sequences of PrP genes. For example, a chicken, bovine, sheep, rat and mouse PrP gene are disclosed and published within Gabriel et al. (1992) *Proc. Natl. Acad. Sci. USA* *89*:9097-9101. The sequence for the Syrian hamster is published in Basler et al. (1986) *Cell* *46*:417-428. The PrP gene of sheep is published by Goldmann et al. (1990) *Proc. Natl. Acad. Sci. USA* *87*:2476-2480. The PrP gene sequence for bovine is published in Goldmann et al. (1991) *J. Gen. Virol.* *72*:201-204. The sequence for chicken PrP gene is published in Harris et al. (1991) *Proc. Natl. Acad. Sci. USA* *88*:7664-7668. The PrP gene sequence for mink is published in Kretzschmar et al. (1992) *J. Gen. Virol.* *73*:2757-2761. The human PrP gene sequence is published in Kretzschmar et al. (1986) *DNA* *5*:315-324. The PrP gene sequence for mouse is published in Locht et al. (1986) *Proc. Natl. Acad. Sci. USA* *83*:6372-6376 . The PrP gene sequence for sheep is published in Westaway et al. (1994) *Genes Dev.* *8*:959-969 . These publications are all incorporated herein by reference to disclose and describe the PrP gene and PrP amino acid sequences.

## Standardized Prion Preparation

[0088] Standardized prion preparations may be produced in order to test assays of the invention and thereby improve the reliability of the assay. Although the preparation can be obtained from any animal it is preferably obtained from a host animal which has brain material containing prions of a test animal. For example, a transgenic mouse containing a bovine prion protein gene can produce bovine prions and the brain of such a mouse can be used to create a standardized bovine prion preparation. Further, in that the preparation is to be a "standard" it is preferably obtained from a battery (*e.g.*, 100; 1,000, or more animals) of substantial identical animals. For example, 100 mice all containing a very high copy number of bovine PrP genes (all polymorphisms and mutations) would spontaneously develop disease and the brain tissue from each could be combined to make a useful standardized prion preparation. Standardized prion preparations are described and disclosed in U.S. Patent 5,908,969 issued June 1, 1999

and U.S. Patent 6,020,537 issued February 1, 2000, both of which are incorporated herein in their entirety.

**[0089]** Standardized prion preparations can be produced using any of modified host mammals of the type described above. For example, standardized prion preparations can be produced using mice, rats, rabbits, hamsters, or guinea pigs which are genetically modified so that they are susceptible to infection with prions which prions would generally only infect genetically diverse species such as a cow, sheep, deer or horse and which modified host mammals will develop clinical signs of CNS dysfunction within a period of time of 350 days or less after inoculation with prions. The most preferred host mammal is a mouse in part because they are inexpensive to use and because a greater amount of experience has been obtained with respect to production of transgenic mice than with respect to the production of other types of host animals. Details regarding making standardized prion preparation are described in U.S. Patents 6,008,435 and 6,020,537, both of which are incorporated herein by reference.

**[0090]** Once an appropriate type of host is chosen, such as a mouse, the next step is to choose the appropriate type of genetic manipulation to be utilized to produce a standardized prion formulation. For example, the mice may be mice which are genetically modified by the insertion of a chimeric gene of the invention. Within this group the mice might be modified by including high copy numbers of the chimeric gene and/or by the inclusion of multiple promoters in order to increase the level of expression of the chimeric gene. Alternatively, hybrid mice of the invention could be used wherein mice which have the endogenous PrP gene ablated are crossed with mice which have a bovine PrP gene inserted into their genome. There are, of course, various subcategories of such hybrid mice. For example, the bovine PrP gene may be inserted in a high copy number an/or used with multiple promoters to enhance expression. In yet another alternative the mice could be produced by inserting multiple different PrP genes into the genome so as to create mice which are susceptible to infection with a variety of different prions, i.e., which generally infect two or more types of test animals. For example, a mouse could be created which included a chimeric gene including part of the sequence of a cow, a separate chimeric gene which included part of the sequence of a deer, and still another chimeric gene which included part of the sequence of a sheep. If all three different types of chimeric genes were inserted into the genome of the mouse the mouse would be susceptible to infection with prions which generally only infect a cow, deer and sheep.

**[0091]** After choosing the appropriate mammal (*e.g.*, a mouse) and the appropriate mode of genetic modification (*e.g.*, inserting a chimeric PrP gene such as MBo2M) the next step is to

produce a large number of such mammals which are substantially identical in terms of genetic material related to prions. More specifically, each of the mice produced will include an identical chimeric gene present in the genome in substantially the same copy number. The mice should be sufficiently identical genetically in terms of genetic material related to prions that 95% or more of the mice will develop clinical signs of CNS dysfunction within 350 days or less after inoculation and all of the mice will develop such CNS dysfunction at approximately the same time *e.g.*, within ± 30 days of each other.

**[0092]** Once a large group *e.g.*, 50 or more, more preferably 100 or more, still more preferably 500 or more of such mice are produced. The next step is to inoculate the mice with prions which generally only infect a genetically diverse mammal *e.g.*, prions from an bovine such as a sheep, cow, deer or horse. The amounts given to different groups of mammals could be varied. After inoculating the mammals with the prions the mammals are observed until the mammals exhibit symptoms of prion infection *e.g.*, clinical signs of CNS dysfunction. After exhibiting the symptoms of prion infection the brain or at least a portion of the brain tissue of each of the mammals is extracted. The extracted brain tissue is homogenized which provides the standardized prion preparation.

**[0093]** As an alternative to inoculating the group of transgenic mice with prions from a genetically diverse animal it is possible to produce mice which spontaneously develop prion related diseases. This can be done, for example, by including extremely high copy numbers of a cow PrP gene into a mouse genome. When the copy number is raised to, for example, 100 or more copies, the mouse will spontaneously develop clinical signs of CNS dysfunction and have, within its brain tissue, prions which are capable of infecting humans. The brains of these animals or portions of the brain tissue of these animals can be extracted and homogenized to produce a standardized prion preparation.

**[0094]** The standardized prion preparations can be used directly or can be diluted and titered in a manner so as to provide for a variety of different positive controls. More specifically, various known amounts of such standardized preparation can be used to inoculate a first set of transgenic control mice. A second set of substantially identical mice are inoculated with a material to be tested i.e., a material which may contain prions. A third group of substantially identical mice are not injected with any material. The three groups are then observed. The third group, should, of course not become ill in that the mice are not injected with any material. If such mice do become ill the assay is not accurate probably due to the result of producing mice which spontaneously develop disease. If the first group, injected with a standardized

preparation, do not become ill the assay is also inaccurate because the mice have not been correctly created so as to become ill when inoculated with prions which generally only infect a genetically diverse mammal. However, if the first group does become ill and the third group does not become ill the assay can be presumed to be accurate. Thus, if the second group does not become ill the test material does not contain prions and if the second group does become ill the test material does contain prions.

[0095] By using standardized prion preparations of the invention it is possible to create extremely dilute compositions containing the prions. For example, a composition containing one part per million or less or even one part per billion or less can be created. Such a composition can be used to test the sensitivity of the antibodies, assays and methods of the invention in detecting the presence of prions.

[0096] Standardized prion preparations are desirable in that they will include a constant amount of prions and are extracted from an isogeneic background. Accordingly, contaminates in the preparations will be constant and controllable. Standardized prion preparations will be useful in the carrying out of and calibration of bioassays in order to determine the presence, if any, of prions in various pharmaceuticals, products produced by using bovines including foods, cosmetics, etc.

## Utility

[0097] As indicated above and described further below in detailed examples it is possible to use the methodology of the invention to create a wide range of different antibodies. i.e., antibodies having different specific features. For example, antibodies can be created which bind only to a $PrP^C$ protein naturally occurring within a single bovine species or $PrP^C$ from human and not bind to a $PrP^C$ protein naturally occurring within other species. Further, the antibody can be designed so as to bind only to a non-infectious form of human or a bovine prion protein (*e.g.*, $PrP^C$) and not bind to an infectious form (*e.g.*, $PrP^{Sc}$). A single antibody or a battery of different antibodies can then be used to create an assay device. Such an assay device can be prepared using conventional technology known to those skilled in the art. The antibody can be purified and isolated using known techniques and bound to a support surface using known procedures. The resulting surface having antibody bound thereon can be used to assay a sample *in vitro* to determine if the sample contains one or more types of antibodies.

**Treatment--General**

[0098] An assay of the invention can use all or any of three basic types of treatment such as those discussed herein. The treatments can include (1) pretreatment, (2) unfolding treatment and (3) hydrolysis treatment. In general, the conditions for pretreatment are gentle, those for unfolding treatment moderate and those for hydrolysis treatment are harsh. Each type of treatment can employ the same means (e.g. proteases, time, pH, temperature, etc.) but employs each to a different degree, e.g. higher concentration, longer time, higher temperature. However, the hydrolysis treatment must employ a compound which selectively hydrolyzes only the non-disease conformation and not the disease conformation.

**Pretreatment**

[0099] Before carrying out treatment or antibody testing of the sample it may be desirable to subject the sample to pretreatment. The pretreatment is carried out in order to destroy or remove unrelated proteins as well as some of the non-disease form of the protein present within the sample. Examples of pretreatment methodology include producing a column which includes antibodies bound to support surfaces which antibodies bind to the non-disease conformation of the protein thereby removing as much of the non-disease conformation of the proteins possible. Antibodies which bind unrelated but common proteins can also be used. Alternatively, the sample can be subjected to physical treatment such as long term hydrostatic pressure or temperature alone or in combination with chemicals such as acids or alkalines as indicated above to destroy proteins present in the sample which proteins are not related to those being assayed for or are in the non-disease conformation. In some instances proteins in the non-disease and disease conformation will be destroyed. However, a higher relative percentage of the proteins in the non-disease conformation will be destroyed because these proteins are initially in a looser conformation which is more vulnerable to destruction. Thus, the pretreatment methodology results in a sample which includes a relatively lower concentration of the non-disease conformation of the protein relative to the concentration of the disease conformation of the protein. Further, the pretreated sample will have a lower concentration of unrelated proteins. This increases the sensitivity of the assay making it possible to detect lower concentrations of the disease conformation of the protein. Removal of proteins is preferred over destruction of such in that destruction will decrease sensitivity if the disease conformation is destroyed. A particularly useful pretreatment method is disclosed in U.S. Pat. No. 5,977,324, issued Nov. 2, 1999.

**Unfolding Treatment**

**[00100]** The unfolding treatment denatures the protein but does not hydrolyze proteins of interest and can include exposing the proteins to any physical and/or chemical means which causes the protein which is originally present in a tightened, disease related conformation (e.g., PrP.sup.Sc) to assume a more relaxed conformation which has a higher degree of binding affinity for any binding partner such as antibodies (e.g., expose an N-terminal epitope of PrP.sup.Sc). In general, the unfolding treatment involves subjecting the protein to some means which causes epitopes on the protein which were not previously exposed or partially exposed to become exposed or become more exposed so that an antibody or other binding partner can more readily bind to the newly exposed epitope.

**[00101]** Methods used for unfolding treatment may include: (1) physical, such as hydrostatic pressure or temperature, (2) chemical, such as acidic or alkaline pH, chaotropic salts, denaturing detergents, guanidine hydrochloride and proteinases such as Proteinase K and (3) combinations of above.

**[00102]** The treatment time will vary depending on the treatment used but should be carried out for sufficient time to obtain the desired effect, e.g. for unfolding treatment to expose new binding sites but not so long as to completely denature or hydrolyze the protein. When carrying out unfolding treatment on PrP proteins without chemical treatment the temperature is raised to about 40°C. to about 80°C. for a time sufficient to obtain the desired amount of unfolding of $PrP^{Sc}$. The temperature can be lower and the time shorter if the pH is raised to 12 or 13.

**Hydrolysis Treatment**

**[00103]** The hydrolysis treatment is a lytic treatment which is the most important treatment method used in one embodiment of the assays of the invention. After a sample has been subjected to the pretreatment treatment it is subjected to the hydrolysis treatment. This treatment will destroy or hydrolyze all or substantially all protein in the sample which is in the non-disease conformation and not hydrolyze (but unfold) the protein in the disease conformation. The hydrolysis treatment is prferably via an enzyme such as a hydrolase that acts on peptide bonds, preferably a neutral protease, more preferably a metalloendopeptidase, and most preferably dispase or leucostoma peptidase A. The proteases used in the method of the invention may be used alone, in combination, or in conjunction with enzymes having similar but distinct activity such as a carbohydrase, e.g. collagenase, amylase, or alkaline serine protease. The concentration of the treating compounds as well as the time and

temperature will vary with the protein being treated and end result to be obtained. For example, with PrP the treatment is carried out to hydrolyze all or substantially all non-$PrP^C$ present, but not hydrolyze (but unfold) $PrP^{Sc}$ present. The object of this treatment is to hydrolyze as much non-disease protein as possible (preferably all) while hydrolyzing as little (preferably none) disease related protein as possible. The treatment is preferably designed such that it can be quickly and completely stopped at any given time. For example, hydrolysis of $PrP^C$ with dispase or other related proteases can be stopped by adding EDTA.

## Assays

**[00104]** One embodiment of the invention features assays allowing detection of $PrP^{Sc}$ in a human or in a bovine sample by 1) digesting the sample with an enzyme that effectively degrades $PrP^C$ and which denatures $PrP^{Sc}$, or alternatively by successive treatment with an enzyme that degrades $PrP^C$ (but not PrPSc) and then an enzyme which denatures $PrP^{Sc}$ and 2) detecting the denatured $PrP^{Sc}$ using an antibody of the present invention. For example, a sample containing bovine PrP proteins (i.e., $PrP^C$ and $PrP^{Sc}$) can be subjected to hydrolysis by the use of proteinase K (PK) digestion. The use of such at appropriate concentration, times and temperature will digest $PrP^C$ but not $PrP^{Sc}$. Following digestion with proteinase K, the sample is further digested to denature the $PrP^{Sc}$, and the sample is contacted with an antibody of the present invention under suitable binding conditions. Preferably, the antibody is bound to a substrate and can be positioned such that the sample can be easily contacted with the substrate material having the antibody bound thereon. If material binds to the antibodies on the substrate the presence of infectious $PrP^{Sc}$ is confirmed.

**[00105]** In another embodiment, a sample to be tested is divided into two portions, and one is digested to denature any $PrP^{Sc}$ in the sample without destroying the $PrP^C$ in the sample. Both portions are contacted with an antibody of the invention, which will bind to $PrP^C$ in the untreated portion and both $PrP^C$ and $PrP^{Sc}$ in the treated portion. Levels of $PrP^C$ or $PrP^C$ + $PrP^{Sc}$ are detected and the amount of $PrP^{Sc}$ in the sample determine from the difference in detectable signal between the two samples.

**[00106]** In some embodiments of the invention it may be desirable to use antibodies of the invention in a sandwich type assay. More particularly, the antibody of the invention may be bound to a substrate support surface. The denatured sample to be tested is contacted with the support surface under conditions which allow for binding. Thereafter, unreacted sites are blocked and the surface is contacted with a detection antibody. The detection antibody may

provide for an additional level of specificity, e.g. by specifically binding to PrP of a mammal of interest, to a specific domain of interest, or broadly cross-reactive to mammalian PrP proteins. Alternatively, the detection antibody may be non-specific.

**[00107]** The detection antibody is linked to a detectable label. The detection antibody with detectable label is allowed to bind to any PrP bound to the antibodies on the support surface. If binding occurs the label can be made to become detectable such as by generating a color thereby indicating the presence of the label which indirectly indicates the presence of PrP within the sample. The assay can detect denatured PrP present in an amount of 1 part per million or less, even one part per billion or less. The PrP may be present in a source selected from the group consisting of (a) a pharmaceutical formulation containing a therapeutically active component extracted from an animal source, (b) food products, (c) an organ, tissue, body fluid or cells extracted from a human source, (d) an animal-based product such as injectables, orals, creams, suppositories, and intrapulmonary delivery formulations, (e) a cosmetic, and (f) a pharmaceutically active compound extracted from a mammalian cell culture.

## EXAMPLES

**[00108]** The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

## EXAMPLE 1

**[00109]** Immunization and Hybridoma Production: PrP ablated FVB female mice were immunized with either recombinant bovine PrP 102-241 protein (recBoPrP 102-241) or recombinant human PrP 90-231 protein (recHuPrP90-231) as follows. Both recBoPrP102-241 and recHuPrP90-231 were prepared in RIBI adjuvant (Corixa, Corp.) as suggested by the manufacturer at a concentration of 1 mg/mL. Animals were immunized by subcutaneous (SC) injection of 100 μg of antigen followed by two booster immunizations at 14-day intervals. In some fusions experiments either native recBoPrP 102-241 or recHuPrP90-231 protein was

used, where as in other fusions the protein was first boiled in 0.2% SDS before suspension in adjuvant. To serve as a guide for selecting mice for subsequent cell fusion experiments, serum was obtained from each animal and analyzed for anti-prion antibody activity using an enzyme-linked immunosorbent assay (ELISA) and by Immunoblot experiments. Animals with strong immune responses were chosen for use in subsequent hybridoma production.

**[00110]** Hybridoma Production - Cell Fusion: Chemical cell fusions were performed as previously described. Briefly, P3X63AG8U.1 myeloma cells were maintained in Dulbecco's modified Eagles Media with high glucose and L-glutamine (without sodium pyruvate) (Gibco-BRL #11965-092) supplemented with 10% fetal calf serum (Gibco-BRL #10082-147), Na pyruvate (2 mM) and penicillin/streptomycin. This media was adjusted to 15% FCS and 1 mM non-essential amino acids to maintain subsequent hybridoma cells. Splenocytes were dispersed using a stainless steel mesh, the erythrocytes lysed by incubating in 0.75% $NH_4Cl$ for 5 min on ice. Washed myeloma cells (viability greater than 99%) were mixed with splenocytes (a ratio of 5 splenocytes to 1 myeloma cell) and the cells centrifuged at 2000 rpm for 5 min. The supernatant was removed, the pellet gently disrupted and 1 mL per $10^8$ cells of a 50% polyethylene glycol solution slowly added. The cell mixture was then centrifuged again (500 x g) for 5 min, the PEG solution removed and the cell pellet slowly resuspended and incubated for 20 min at 37° C. Finally, the cells were centrifuged (500 x g, 5 min), resuspended in growth medium and incubated overnight at 37°C in an 8% $CO_2$ atmosphere. The next day the cells were resuspended in growth medium containing hypoxanthine, aminoptrin and thymidine (HAT-medium) and dispersed over ten 96-well microculture dishes containing irradiated 3T3 fibroblast cells acting as a feeder layer. Following growth for 10-14 days, media from those wells observed to have colonies of hybridoma cells was screened for anti-prion antibodies. Monoclonal antibodies detected with this method are listed in Table 3.

**[00111]** Screening: A multistep screening schedule (outlined below) was employed incorporating a direct binding ELISA, a newly developed double capture immunoassay (DCI) -and Immunoblot analyses on normal brain material from various species.

**[00112]** Antibody producing hybridomas were cloned at least 3 times by limited dilution and an ascites tumor induced by intraperitoneal injection of approximately 5 x $10^6$ hybridoma cells into BALB/c mice and into PrP ablated Severe Combined Immunodeficient (SCID) mice. The ascites fluid was harvested and the antibody purified by affinity chromatography on a Protein – G column.

[00113] ELISA Methods: A newly developed rapid double capture immunoassay (DCI) was developed and validated. This assay allows for early (at the first step in the screening process) identification of monoclonal antibodies that are useful as capture reagents. The DCI is shown in **Figure 1**. Microtiter wells of a black fluorescence assay plate are first coated by passive absorbtion with goat anti-mouse IgG Fc specific antibody (Antibody #1) (Chemicon #AP127) (50 µL/well of a 2µg/mL solution in carbonate coating buffer, 0.1 M carbonate, pH 8.6) by incubation at 37° C for 2 h. Reactive sites on the plastic are then blocked by addition of a solution of 5% non-fat dry milk (NFDM) in Tris-Buffered Saline-tween (TBST, 0.01 M Tris-base, 0.8%NaCl, 0.05 % tween-20, pH 7.4) and the plates incubated for 1 hr at 37 °C. Hybridoma supernatants are then added and the plate incubated overnight at 4°C. (This step allows for attachment of any mouse monoclonal antibodies in the supernatants to the immobilized antibody #1). The supernatant is removed, the plate washed and blocked a second time using 250 µL/well of a 1:1000 dilution of pure mouse IgG (Chemicon #PP54) in 5% NFDM blocking buffer. This second block is critical in order to reduce background fluorescence resulting from binding of not occupied combining sites of the goat-anti mouse antibody (Antibody #1). Next, the plates are decanted and either recBoPrP102-241 or recHuPrP90-231 at a concentration of 2 µg/mL in blocking buffer is added, the plates sealed and then they are incubated at 37°C for 2 h. The plates are then washed 3 times incorporating a 1 min soak per cycle, patted dry and 200 µL/well of Europium conjugated anti-prion antibody HuM-P(HuM-P-Eu) at a concentration of 0.25 µg/mL in assay buffer is added and the plates incubated for 2 h at 37 °C. Finally, the plates are washes and 200 µL/well of enhancement solution (Perkin Elmer #1244-105) is added, the plates incubated for 10 min and fluorescence (time-resolved) intensity measured using a Packard 'Discovery' fluorometer. The direct binding ELISA is as previously described (Williamson paper). Isotype analysis was performed using isotype specific alkaline phosphatase conjugated anti-mouse Immunoglobulin (SouthernBiotech Associates Inc., Biringham, AL) in an ELISA format and secondly, using a one-step lateral-flow system, IsoStrip (Roche Diagnostics Corp., Indiannapolis, IN) .

[00114] Peptide assays: The ability of F4-31, F4-30, F4-7, F20-80, and F20-130a to bind the following peptides was evaluated using the direct binding ELISA where the peptides were used as antigens to coat the microtiter wells. A series of peptides, 12 amino acids in length with a 7 amino acid overlap, representing the bovine PrP molecule from amino acid 25 to 241 was obtained from Sigma Genysis (Woodlands, TX). Three Lysine residues were added to the

amino end of each peptide to improve solubility and a single molecule of biotin was added to the amino terminal Lysine. These peptides are listed in Table 1.

Table 1

N-terminal biotinylated 12 amino acid peptides with an 8 amino acid overlap spanning the bovine PrP molecule.

| Peptide No | Peptide Name | Sequence (N- to C-Term) | SEQ ID NO. |
|---|---|---|---|
| 1 | RecBoPrP 25-36 | KKRPKPGGGWNT | SEQ ID NO:1 |
| 2 | RecBoPrP 30-41 | PGGGWNTGGSRY | SEQ ID NO:2 |
| 3 | RecBoPrP 35-46 | NTGGSRYPGQGS | SEQ ID NO:3 |
| 4 | RecBoPrP 40-51 | RYPGQGSPGGNR | SEQ ID NO:4 |
| 5 | RecBoPrP 45-56 | GSPGGNRYPPQG | SEQ ID NO:5 |
| 6 | RecBoPrP 50-61 | NRYPPQGGGGWG | SEQ ID NO:6 |
| 7 | RecBoPrP 52-62 | QGGGGWGQPHGG | SEQ ID NO:7 |
| 8 | recBoPr60-71 | WGQPHGGGWGQP | SEQ ID NO:8 |
| 9 | RecBoPrP 65-76 | GGGWGQPHGGGW | SEQ ID NO:9 |
| 10 | RecBoPrP 70-81 | QPHGGGWGQPHG | SEQ ID NO:10 |
| 11 | RecBoPrP 76-86 | GWGQPHGGGWGQ | SEQ ID NO:11 |
| 12 | RecBoPrP 80-91 | HGGGWGQPHGGG | SEQ ID NO:12 |
| 13 | RecBoPrP 85-96 | GQPHGGGWGQPH | SEQ ID NO:13 |
| 14 | RecBoPrP 90-101 | GGWGQPHGGGGW | SEQ ID NO:14 |
| 15 | RecBoPrP 95-106 | PHGGGGWGQGGT | SEQ ID NO:15 |
| 16 | RecBoPrP 100-111 | GWGQGGTHGQWN | SEQ ID NO:16 |
| 17 | RecBoPrP 105-116 | GTHGQWNKPSKP | SEQ ID NO:17 |
| 18 | RecBoPrP 110-121 | WNKPSKPKTNMK | SEQ ID NO:18 |
| 19 | RecBoPrP 115-126 | KPKTNMKHVAGA | SEQ ID NO:19 |
| 20 | RecBoPrP 120-131 | MKHVAGAAAAGA | SEQ ID NO:20 |
| 21 | RecBoPrP 125-136 | GAAAAGAVVGGL | SEQ ID NO:21 |
| 22 | RecBoPrP 130-140 | GAVVGGLGGYML | SEQ ID NO:22 |
| 23 | RecBoPrP 135-146 | KGLGGYMLGSAMS | SEQ ID NO:23 |
| 24 | RecBoPrP 128-139 | MLGSAMSRPLIH | SEQ ID NO:24 |
| 25 | RecBoPrP 133-144 | MSRPLIHFGSDY | SEQ ID NO:25 |
| 26 | RecBoPrP 138-149 | IHFGSDYEDRYY | SEQ ID NO:26 |
| 27 | RecBoPrP 143-154 | DYEDRYYRENMH | SEQ ID NO:27 |
| 28 | RecBoPrP 148-159 | YYRENMHRYPNQ | SEQ ID NO:28 |
| 29 | RecBoPrP 153-164 | MHRYPNQVYYRP | SEQ ID NO:29 |
| 30 | RecBoPrP 158-169 | NQVYYRPVDQYS | SEQ ID NO:30 |
| 31 | RecBoPrP 163-174 | RPVDQYSNQNNF | SEQ ID NO:31 |
| 32 | RecBoPrP 168-179 | YSNQNNFVHDCV | SEQ ID NO:32 |
| 33 | RecBoPrP 173-184 | NFVHDCVNITVK | SEQ ID NO:33 |
| 34 | RecBoPrP 178-189 | CVNITVKEHTVT | SEQ ID NO:34 |
| 35 | RecBoPrP 183-194 | VKEHTVTTTTKG | SEQ ID NO:35 |
| 36 | RecBoPrP 188-199 | VTTTTKGENFTE | SEQ ID NO:36 |
| 37 | RecBoPrP 193-204 | KGENFTETDIKM | SEQ ID NO:37 |
| 38 | RecBoPrP 198-209 | TETDIKMMERVV | SEQ ID NO:38 |
| 39 | RecBoPrP 203-213 | KMMERVVEQMCI | SEQ ID NO:39 |
| 40 | RecBoPrP 208-219 | VVEQMCITQYQR | SEQ ID NO:40 |
| 41 | RecBoPrP 213-224 | CITQYQRESQAY | SEQ ID NO:41 |
| 42 | RecBoPrP 218-231 | QRESQAYYQRGA | SEQ ID NO:42 |

[00115] Each of the sequences were biotinylated at their amino terminal end, and subjected to amidation on the C-terminal.

[00116] The peptides listed in Table 2 corresponding to longer fragments of the bovine PrP molecule also were used to evaluate antibody F-31 binding in a direct ELISA. No binding of F4-31 to any of these longer peptides was observed.

Table 2: Bovine peptides

| Sequence Number | Peptide Sequence |
|---|---|
| BoPrP 102-190 | GQGGTHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFSDYE DRYYRENMHRYPNQVYYRPVDQYSNQNNFVHDC (SEQ ID NO:43) |
| Bo PrP 190-242 | CVNITVKEHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQRGAS (SEQ ID NO:44) |
| Hu-PrP 175-230 | FVHDCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGS (SEQ ID NO:45) |
| Bo PrP 186-212 | FVHDCVNITVKEHTVTTTTKGENFTET (SEQ ID NO:46) |
| BoPrP 206-237 | GENFTETDIKMMERVVEQMCITQYQRESQAYY (SEQ ID NO:47) |
| Bo PrP 222-241 | EQMCITQYQRESQAYYQRGA (SEQ ID NO:48) |

Table 3: Characteristics of Monoclonal Antibodies

| mAb | Isotype | Sandwich DELFIA | Western Blot | | | | | | Reduc -tion | GndHCl |
|---|---|---|---|---|---|---|---|---|---|---|
| F4-7 | IgG1 k | + | Mo | SHa | - | Bo | Ov | MD | S | ND |
| F4-30 | IgG1 k | + | Mo | SHa | - | Bo | Ov | MD | S | ND[4] |
| F4-31 | IgG1 k | + | Mo | SHa | - | Bo | Ov | MD | S[2] | R |
| F20-29 | IgG1 k | + | Š | Š | Hu | Š | Š | Š | S | S |
| F20-49 | IgG1 k | + | Š | SHa | Hu | Š | Š | Š | R[3] | S |
| F20-80 | IgG1 k | - | Mo | SHa | Hu | Bo | Ov | MD | S | S |
| F20-89a | IgG1 k | + | Š | Š | Hu | Š | Ov | MD | R | R |
| F20-108a | IgG1 k | + | Mo | SHa | Hu | w[1] | Ov | MD | S | S |
| F20-130a | IgG1 k | + | Mo | SHa | Hu | Bo | Ov | MD | S | S |

[1] w = weak response; [2] S = sensitive, reduced activity when antigen is reduced, [3]R = resistant, no reduction in activity when antigen is reduced; [4]ND= not dete rmined

[00117] Immunization and Hybridoma Isolation: Strong serum titers were observed by direct ELISA in all the mice following immunization with recHuPrP90-231 and recBoPrP102-241 (**Figure 2A** shows serum titers observed using the recBoPrP102-241 antigen). Immunoblot results (**Figure 2B**) suggested that the sera from these animals contained antibodies that bound PrP from a broad range of animals including mouse, hamster, bovine, sheep, deer, and elk. Identical experiments using sera from mice immunized with human PrP also bound PrP from a broad range of animals including mouse, hamster, human, bovine, sheep, deer and elk. Following cell fusion experiments using splenocytes from mice that were immunized with

either recHuPrP90-231 or recBoPrP102-241, hundreds of microtiter wells contained actively growing hybridoma cells at 10-21 days post fusion were observed. These were screened for the presence of anti-PrP antibodies using the direct binding ELISA. Absorbency measurements greater than 0.3 OD units were observed in approximately 10 to 25% of the wells. Isotype analysis suggested that antibodies of subclass IgM were common and they were not further studied.

**[00118]** The splenocytes from an animal that had received an SDS-denatured immunogen also were used in fusion experiments. Following this fusion substantially fewer growing hybridoma cells (growth in only 63 wells out of 960 wells, ten 96-well microculture plates) were observed. Supernatant from these wells was evaluated in the direct binding ELISA for the presence of anti-PrP antibodies and 7 positive wells (absorbencies greater than 0.3 OD $405_{nm}$ Units) were detected. Isotype analysis of the antibodies in each well indicated that they were of subtype IgG1 with kappa light chains. These supernatants were next evaluated with the DCI and positive responses were observed from six of the supernatants.

**[00119]** Cells from only three of these wells survived the cloning process and continued to produce highly active antibody when analyzed with either the direct binding ELISA, the DCI or by immunoblotting experiments. These clones are referred to as F4-7, F4-30, and F4-31. Each clone was expanded and used to produce ascites fluid.

**[00120]** Injection of the hybridoma cells into BALB/cJ mice resulted in poor ascites production. In contrast, use of PrP ablated SCID mice instead of the BALB/cJ animals resulted in the recovery of large amounts of ascites fluid. Use of Protein-G affinity chromatography resulted in highly purified and active antibodies (**Figure 3A & B**). Antibody F4-31 grew faster than the others and it produce greater amounts of antibody and subsequently it was further characterized.

**[00121]** Antibody Specificity. Monoclonal antibodies F4-7, F4-30 and F4-31 were observed to preferentially bind bovine recBoPrP 102-241 versus recHuPrP30-231 in the CDI (**Figure 4**). Western blot studies using normal brain materials indicates that F4-31 has broad specificity reacting with mouse, hamster, bovine, ovine, deer, and elk, however no binding to human prion was observed in these experiments (**Figure 5**). The limited binding of F4-31 to human PrP in the DCI may reflect changes in antigen presentation between the direct binding ELISA and the DCI, or it may reflect differences in antigen concentration. Results from western blot analyses using mAbs F20-29, F20-49, F20-80, F20-80a, F20-89, and F20-130a are

summarized in Table 3. Antibodies F20-80, F20-108a, and f20-130a have broad specificity where as F20-29, F20-49, and F20-89a demonstrated a more restricted specificity.

Epitope Analysis

[00122] Peptide Binding: Binding assays to a number of short and long peptides were completed in an effort to determine the binding epitope of F4-31 F4-30, F4-7, F20-80, and F20-130a . The peptides in Table 1 are short, 12 amino acid peptides with an eight amino acid overlap that span the bovine PrP molecule from residue 23 to 241. Each peptide has three lysines added to the amino end to improve solubility and a single molecule of biotin was added to the terminal lysine. The peptides were bound to avidin coated microassay plates for the analysis. The amino acid numbering used here is based on the bovine sequence as aligned by Giles and Prusiner (2003)[Giles, K. and Prusiner, S. (2003) Prion protein and dopple (Dpl) sequences. In: *Prion Biology and Diseases*, (S. Prusiner, Editor) Cold Spring Harbor Press, NY, pp. 1023-1032.] Binding of F4-7, F4-30, F4-31, F20-80 ro F20-130a was not observed to any of these peptides (**Figure 6A, results shown only for F4-31**) however strong binding was observed to recBoPrP102-241. In a control experiment using antibody HuM-P (known to bind a sequential epitope) (**Figure 6B**) binding was observed to peptides corresponding to the published sequence for the epitope of HuM-P. No binding of F4-31 to the longer bovine peptides listed in Table 2 was observed. Control experiments using antibodies with known epitopes demonstrated binding to the peptides corresponding to the published epitope for the control antibody.

[00123] Reduction of Disulfide: Since bovine PrP has a single disulfide bond, antibody binding to reduced and non-reduced recHuPrP90-231 and recBoPrP102-241 was evaluated. In these experiments samples treated with (reduced) and without DDT (not reduced) were separated by SDA-PAGE, the prion proteins transferred to filters that were then probed with monoclonal antibody F4-7, F4-30, F4-31 F20-80, and F20-130a followed by treatment with either an alkaline phosphatase or peroxsidase conjugated anti-mouse immunoglobulin and bound antibody was detected using a chemiluminescent substrate. Antibody F4-7, F4-30, F4-31, F20-80, and F20-130a binding was dramatically reduced in the DDT treated sample for each antibody tested (**Figure 7, F4-31 binding shown**, compare lane 5 versus 6) demonstrating that an intact disulfide bond is necessary for binding and suggesting that the binding epitope of these antibodies has a conformational component. Silver staining of these gels (**Figure 7**, lanes 1-2) demonstrates that the reduced recBoPrP102-241 migrates slower in the gel than did the non-reduced protein. Antibody HuM-P is known to bind a sequential epitope distal from the disulfide bond in the PrP. Thus, when antibody HuM-P was used as the probe, as expected,

it bound to both the reduced and not reduced form of the PrP protein demonstrating that the slower migrating protein observed in the reduced lane is indeed PrP102-241(Figure 7, lanes 3-4).

**[00124]** Results from direct binding ELISA experiments were similar to those observed using western blots. In these experiments, recBoPrP102-241 was adjusted to 1 mM DTT and used to coat microtiter wells by incubating the plates overnight. The antigen was then removed, the wells blocked, and a typical ELISA performed. As shown in **Figure 8A**, binding of antibody F4-31, F20-29, F20-80, and F20-130a was substantially less to the reduced PrP versus the not reduced PrP. In contrast, binding of antibodies F20-49 , F20-89a and F20-130a was only slightly reduced (**Figure 8B**). Again, binding of the HuM-P antibody was comparable to either the reduced or not-reduced PrP.

**[00125]** Guanidinium Treatment: Antibody binding to chemically denatured recHuPrP90-231 or to recBoPrP102-241 was evaluated by comparing antibody binding to protein chemically denatured by treatment with 3M guanidinium hydrochloride (GdnHCl). Results shown in **Figure 9A and 9B** demonstrate that no, or only a little loss of antibody for F4-31, F20-29, F20-49, F20-80, F20-89a, F20-108a, and F20-130a binding occurred in denatured protein. Binding of Hum-P to the treated proteins is included as a control.

## EXAMPLE 2

**[00126]** The above shows success in isolating several monoclonal antibodies fragments (Fabs)that capture with high affinity denatured $BoPrP^{Sc}$ but not the native conformation of the same protein in CDI-formatted ELISA. The purified mAb's were used in conformation dependent immunoassay (CDI) as taught in U.S. Patent 5,891,641 to measure bovine, sheep, and deer $PrP^{Sc}$ and to determine the capture affinity constant.

**[00127]** Figure 10 demonstates the increased sensitivity of ungulate prion detection obtained with a CDI assay using mAb F4-31. Capture of $PrP^{Sc}$ from sheep scrapie, chronic wasting disease (CWD) of deer, and BSE by monoclonal antibody F4-31 in sandwich-formated conformation-dependent immunoassay (CDI).

**[00128]** Europium conjugated HuM-P (Eu-HuM-P) was used for detection on the 96-well plates coated either with monoclonal antibody F4-31 or rec(HuM) Fab D18 for comparison. The bars of **Figure 10** represent the average signal obtained from three independent experiments using Eu-HuM-P antibody concentration 0.25 μg/ml. The plates were coated with a 5 μg/ml solution of mAb F4-31 or recFab D18 in 0.2 M phosphate buffer, pH 7.4, overnight at 5°C. After 7

washing steps, the signal was evaluated with Discovery (Packard Inc.) time-resolved fluorescence spectroscopy.

## EXAMPLE 3

**[00129]** Capture affinity constant ($K_D$) of mAb F4-31 determined against different concentrations of recombinant bovine PrP(102-241) is in the low nanomollar range. Capture of recBoPrP(102-241) by monoclonal antibody F4-31 was performed in sandwich-formated conformation-dependent immunoassay (CDI). Nintysix-well microassay plates were coated with the indicated concentration of monoclonal antibody F4-31 in 0.2 M phosphate buffer, pH 7.4, incubated overnight at 5°C, and developed with 0.25 μg/ml of EU-HuM-P. After 7 washing steps, the signal was evaluated with Discovery (Packard Inc.) time-resolved fluorescence spectroscopy. The data points shown in **Figure 11** represent the average obtained from three independent experiments.

## EXAMPLE 4

**[00130]** Sensitivity of monoclonal antibody F4-31 in the CDI for detection of prions in brain homogenates of BSE-infected British cows was measured. In the experiment shown in **Figure 12**, antibody F4-31 was used to capture and Eu-HuM-P was used to detect the Bovine $PrP^{Sc}$. The standard CDI protocol with recFab D18 was used for comparison. The dynamic range of the detection of $BoPrP^{Sc}$ has increased over 1000-fold when compared to recFab D18. Samples containing serial 3-fold dilutions of BSE-infected brain homogenate (5% (w/v)) in 2% Sarcosyl (w/v) were treated with 5 μg/ml of Proteinase K and concentrated with 0.3% (w/v) NaPTA and 1.7 mM $MgCl_2$ prior to CDI. Bovine $PrP^{Sc}$ in native and denatured aliquots was captured with mAb F4-31 or recFab D18–coated ELISA plates and detected with 0.25 μg/ml of Eu-HuM P. After 7 washing steps, the signal was evaluated with Discovery (Packard Inc.) time-resolved fluorescence spectroscopy. The results (**Figure 12**) are expressed in cpm as a difference of the signals from the denatured ($TRF_D$) and native ($TRF_N$) aliquots of each sample. This value is directly proportional to the concentration of $BoPrP^{Sc}$ in the sample.

**[00131]** This certifies that deposits were made at the American Type Culture Collection (ATCC) on July 7, 2005, February 2, 2006 and February 7, 2006. The deposited cell line F4-30 was assigned ATC No. PTA-6838 and the cell line F4-7 was assigned ATC No. PTA-6839. These cell lines will be maintained at the ATCC during the pendency of this application or any utility application filed there off of and during the term of any patent issuing therefrom in

accordance with the rules of the United States Patent and Trademark Office which allows access to the Patent Office during the pendency of the application.

**[00132]** The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

CLAIMS

That which is claimed is:

1. An antibody characterized by its ability to preferentially bind to a denatured protein chosen from denatured human $PrP^C$ and denatured bovine $PrP^C$.

2. The antibody of claim 1, wherein the antibody cross-reacts with $PrP^C$ from other mammals.

3. The antibody of claim 1, wherein the antibody binds to denatured human or to bovine $PrP^{Sc}$ with a binding affinity $K_a$ of $10^7$ l/mol or more and the binding affinity to native bovine $PrP^{Sc}$ is $K_a$ of $10^6$ l/mol or less.

4. The antibody of claim 1, wherein the antibody binds $PrP^C$ and denatured $PrP^{Sc}$ with a binding affinity substantially the same as or greater than an antibody produced by any one of the hybridoma cell lines chosen from F4-7, F4/30, F4-31, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a.

5. The antibody of Claim 1, wherein said antibody is produced by any one of the hybridoma cell lines chosen from F4-31, F4-30, F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a.

6. A method of detecting bovine $PrP^{Sc}$ in a source, comprising:

treating a human or a bovine sample suspected of containing $PrP^{Sc}$ with an enzyme to hydrolyze $PrP^C$ in the sample;

further treating the sample to denature any $PrP^{Sc}$ in the sample; and

contacting the sample suspected of containing $PrP^{Sc}$ with a diagnostically effective amount of an antibody which specifically binds to denatured $PrP^{Sc}$ in the sample wherein the antibody binds $PrP^C$ and denatured $PrP^{Sc}$ with a binding affinity substantially the same as or greater than an antibody produced by any one of the hybridoma cell lines chosen from F4-31, F4-30, F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a; and

determining whether the antibody binds specifically to any material in the sample.

7. The method of claim 6, wherein said antibody is produced by any one of the

hybridoma cell lines chosen from F4-31, F4-30, F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a.

8. An assay, comprising:

a support surface; and

an antibody bound to the surface of the support, the antibody characterized by an ability to bind denatured bovine $PrP^{Sc}$ with a binding affinity of $10^7$ l/mole or more.

9. The assay of claim 8, wherein the antibody is characterized by an ability to bind 50% or more denatured bovine $PrP^{Sc}$ in a liquid flowable sample.

10. The assay of claim 8, wherein a plurality of different antibodies are bound to the support surface and each antibody has a $K_a$ of $10^7$ l/mole or more relative to $PrP^{Sc}$.

11. The assay of claim 8, wherein the tissue is brain tissue and is extracted from a dead bovine or human

12. The assay of claim 8, wherein the antibody binds $PrP^{C}$ and denatures $PrP^{Sc}$ with a binding affinity substantially the same as or greater than an antibody produced by any one of the hybridoma cell lines chosen from F4-31, F4-30, F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a.

13. A method of detecting $PrP^{Sc}$ in a sample, comprising the steps of:

treating a sample obtained from a human or a bovine in a manner which hydrolyzes at least a portion of $PrP^{C}$ in the sample and exposes epitopes on $PrP^{Sc}$ in the sample to provide a treated sample;

contacting the treated sample with a support surface having antibodies bound to the surface wherein the antibodies bind to the exposed epitopes on the $PrP^{Sc}$ with a binding affinity of $10^7$l/mole or greater binding affinity under conditions which allow epitopes on the treated $PrP^{Sc}$ to bind to the antibodies; and

detecting treated $PrP^{Sc}$ bound to the antibodies.

14. The method of claim 13, wherein the antibodies are produced from a hybridoma chosen from F4-31, F4-30, F4-7, F20-29, F20-49, F20-80, F20-89a, F20-108a and F20-130a.

Figure 1: Double Capture Immunoasssay (DCI). Capture antibodies are identified following antigen binding and detection with a recombinant Fab antibody directly conjugated with Europium.

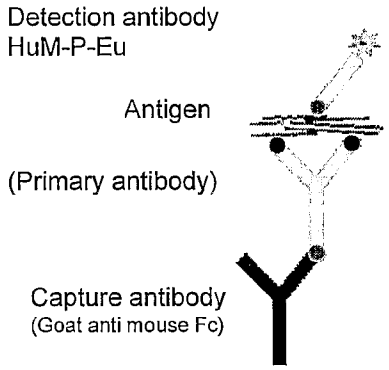


**Figure2A**: DCI analysis of sera from individual mice immunized with rBoPrP102-241

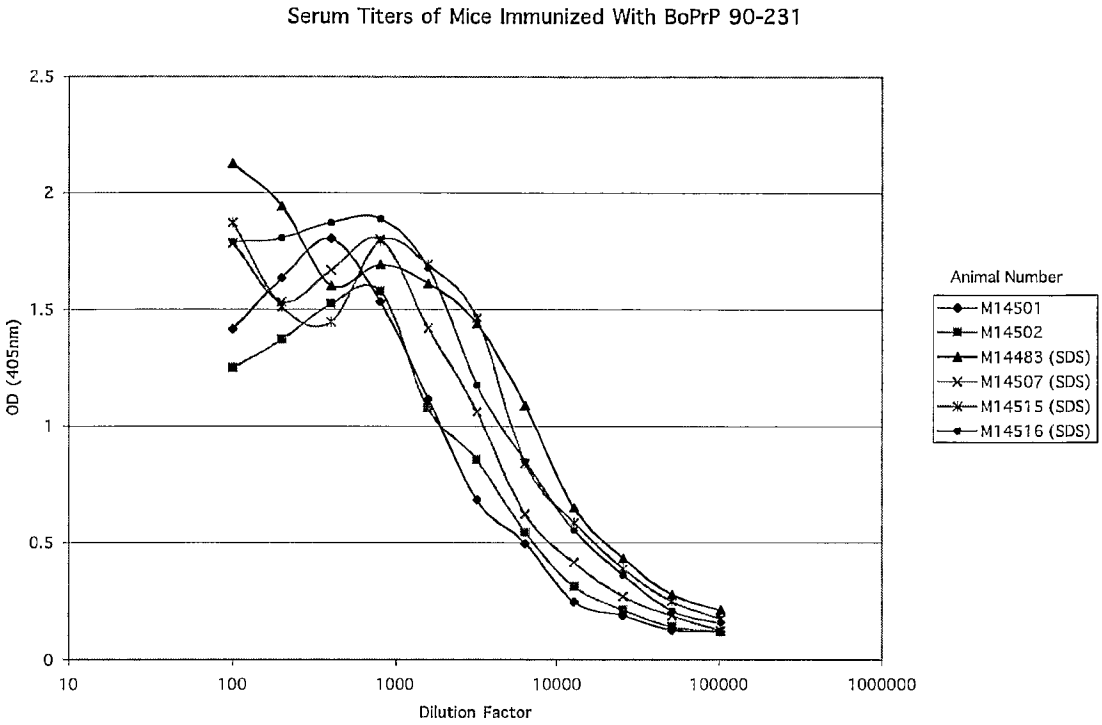


Figure 2B: Western blot analysis of brain homogenate from PrP ablated mouse brain, $PrP^{0/0}$; mouse; Syrian hamster; Huma; bovine; recombinant bovine, recBo102-241, sheep; and mule deer

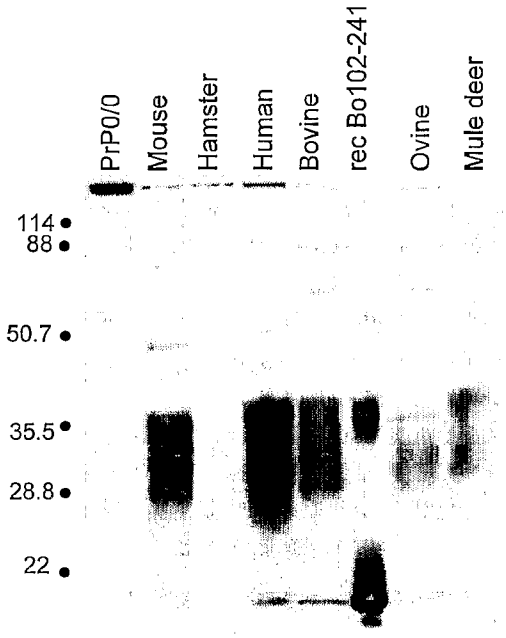


**Figure 3A**: Direct binding ELISA analysis of Protein G purified F4-31 antibody. Microtiter wells were coated with recBoPrP102-241

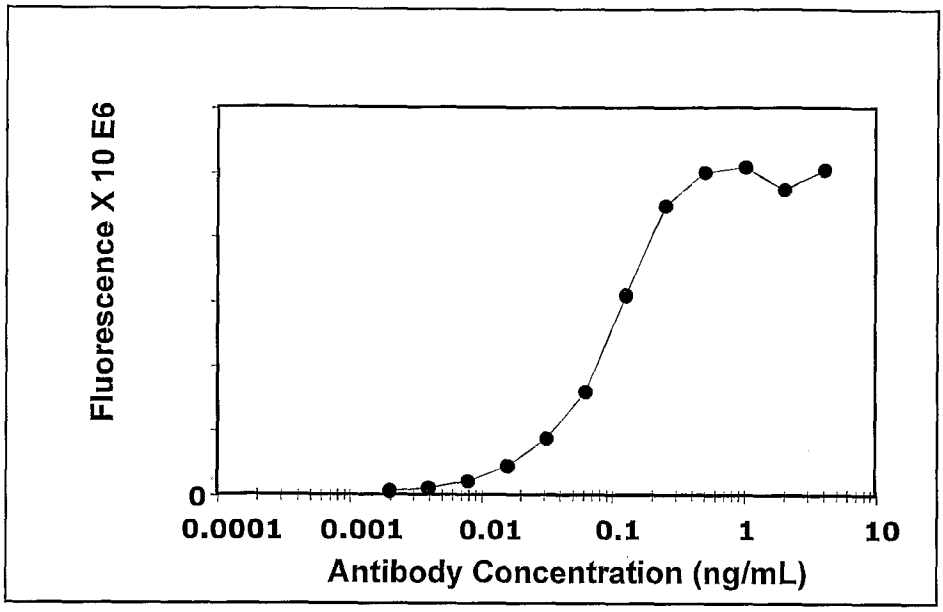



Figure 3B Silver stained SDS-Page gel analysis of Protein-G purified F4-31 antibody. Markers, lane 1; non-reduced sample, lane 2; reduced sample, lane 3.

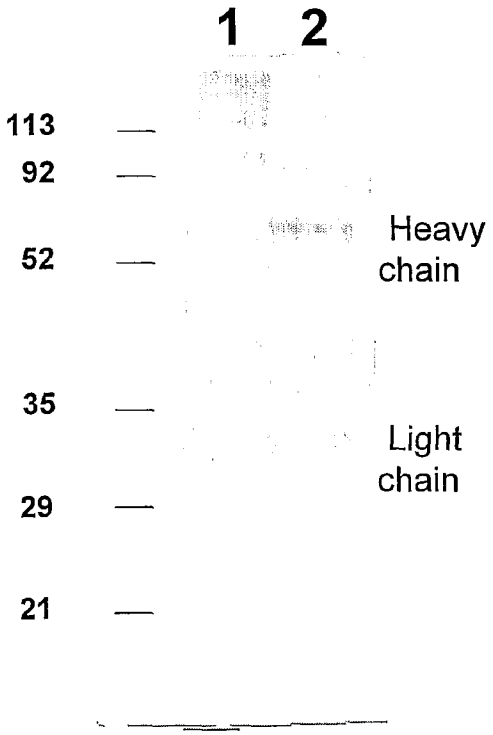


**Figure 4**: DCI titration of F4-31 antibody on Human versus Bovine recPrP.

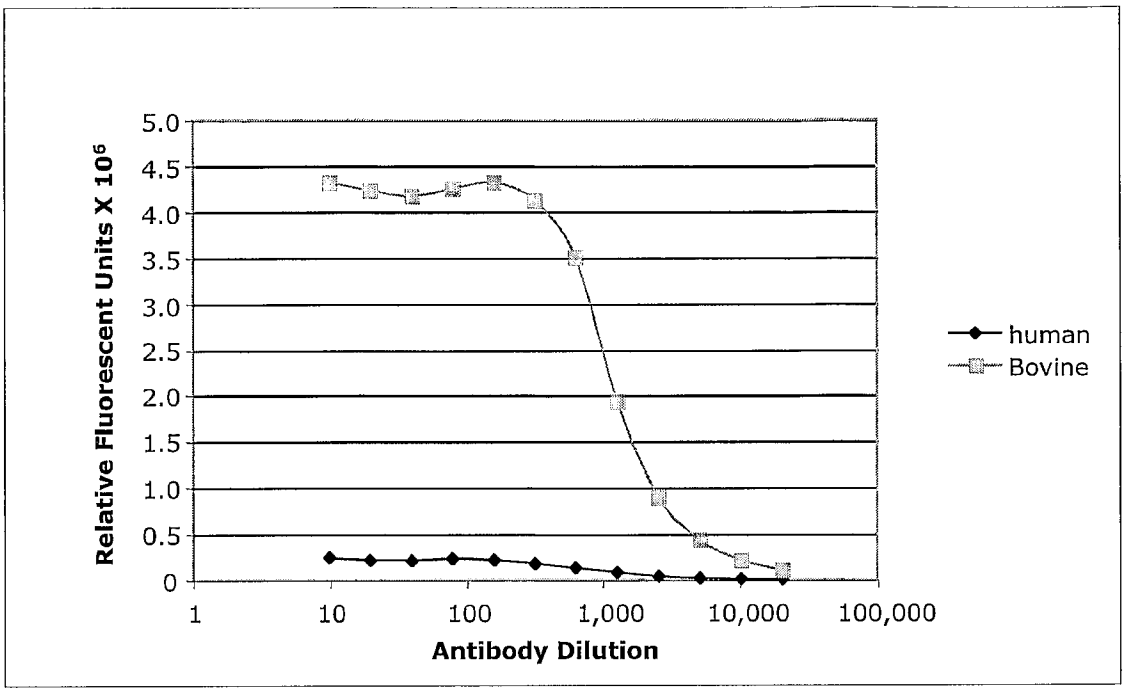


**Figure 5**: Western blot analysis of brain homogenate from PrP ablated mouse brain, $PrP^{0/0}$; mouse, Mo; Syrian hamster, SHa; Human, Hu; bovine, Bo; recombinant bovine, recBo, sheep, Ov; and mule deer, MD detected with monoclonal antibody F4-31

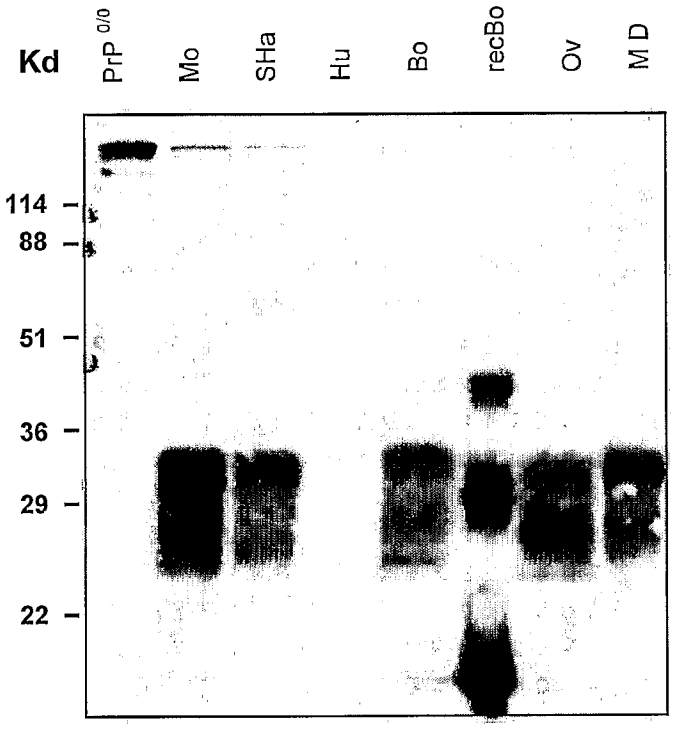


**Figure 6A**: Antibody F4-31 binding to synthetic peptides.

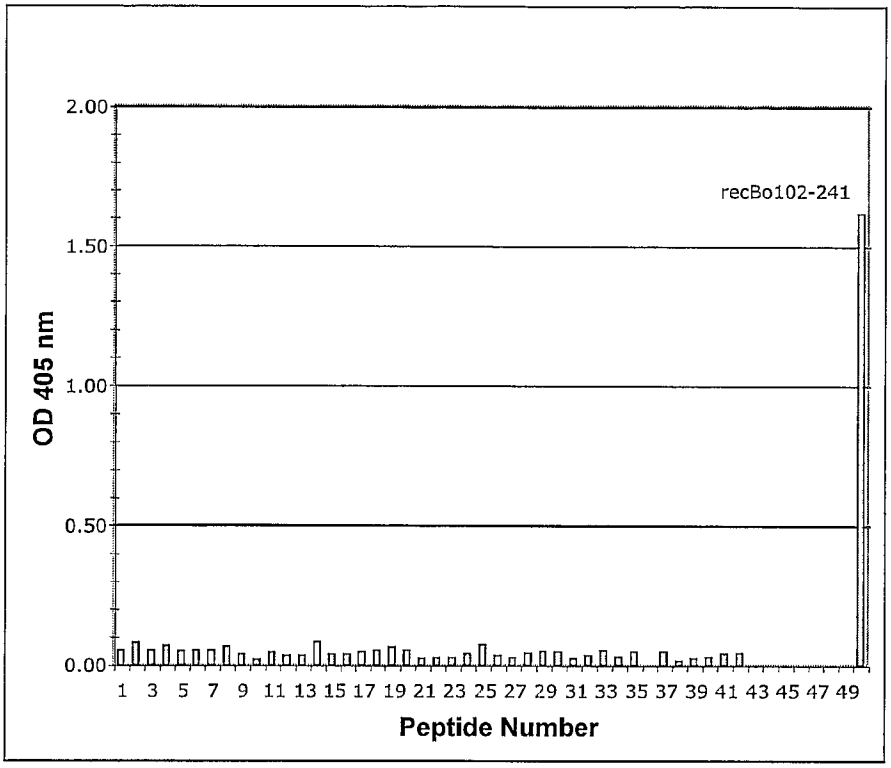



**Figure 6B**: Antibody Hum-P binding to synthetic peptides.

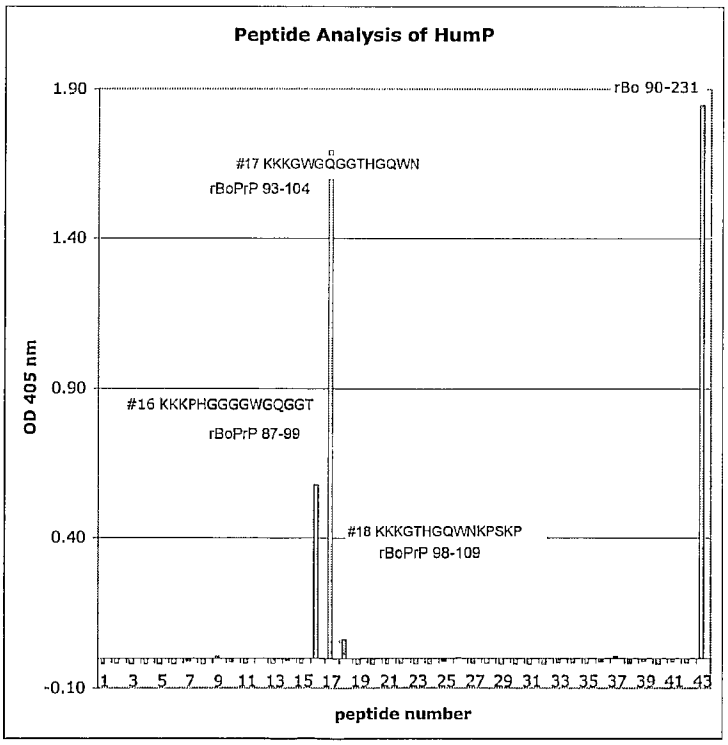


Figure 7: Electrophoresis and western blots of denatured (lanes 1, 3, 5) and non-denatured (lanes 2, 4, 6) rBoPrP90-231 stained with silver stain (lanes 1-2), probed with antibody HumP (lanes 3-4) or probed with monoclonal antibody F4/31 (lanes 5-6).

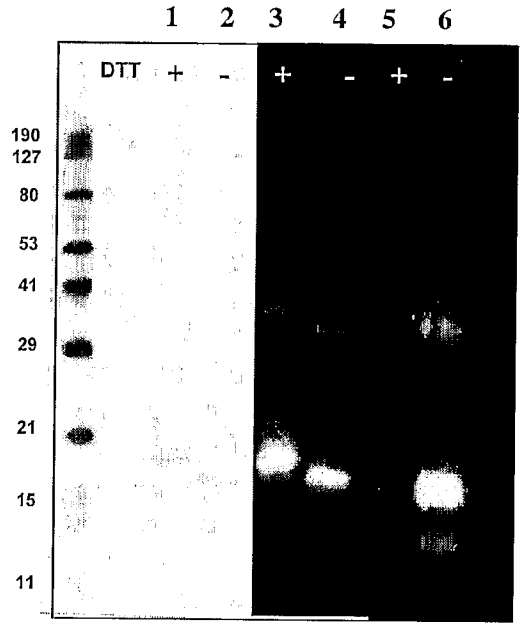
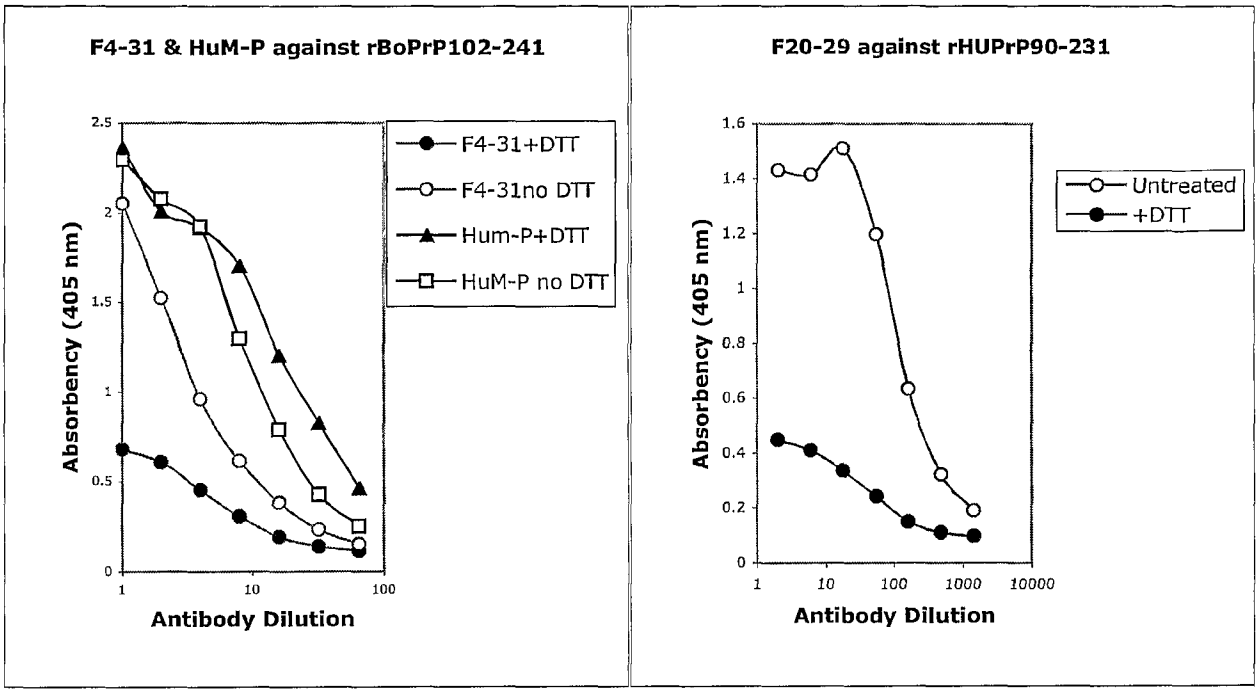


Figure 8A: ELISA titration of monoclonal antibodies F4-31, F20-29, F20-80, F20-130a and HuM-P on reduced (DTT) and non-reduced antigen.



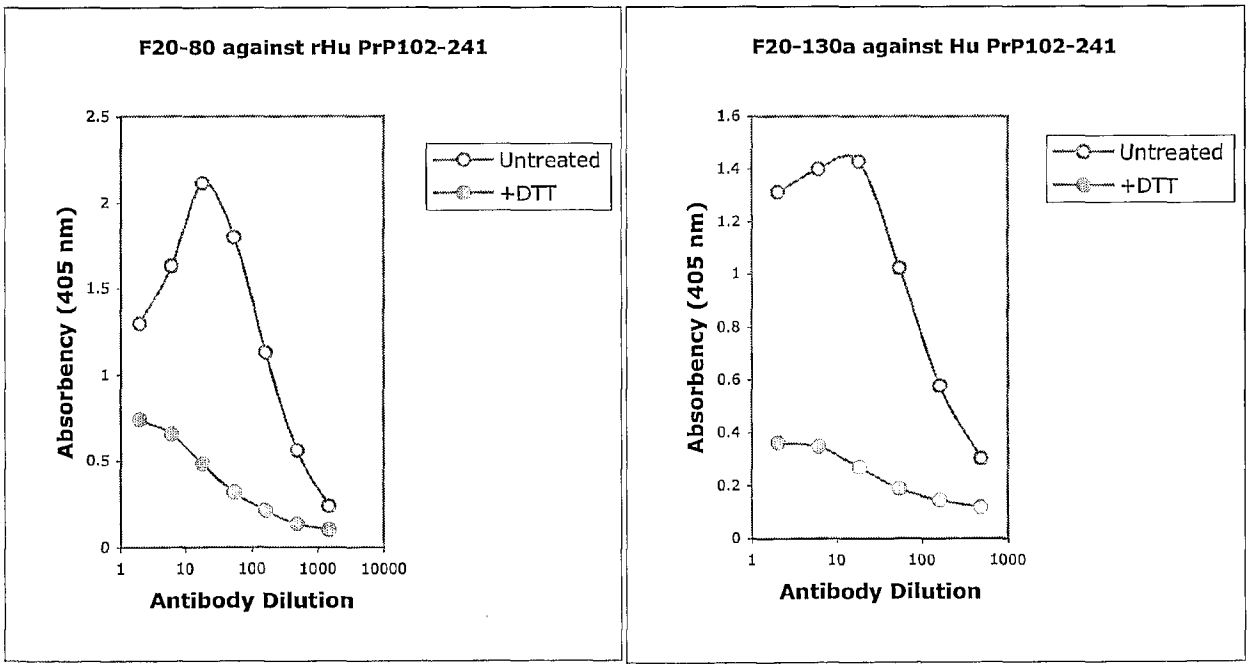
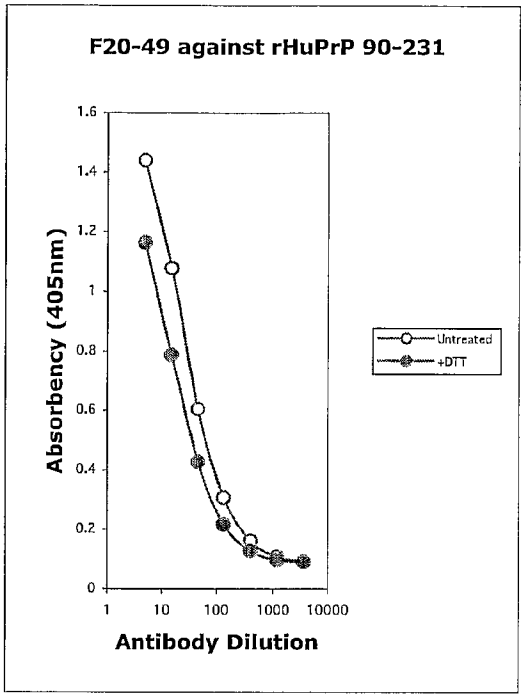



Figure 8B: ELISA titration of monoclonal antibodies F20-49 and F20-89a, on reduced (DTT) and non-reduced antigen.



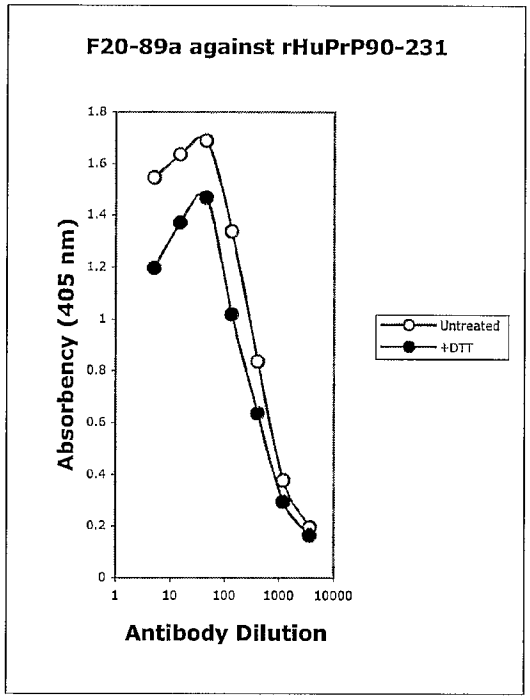
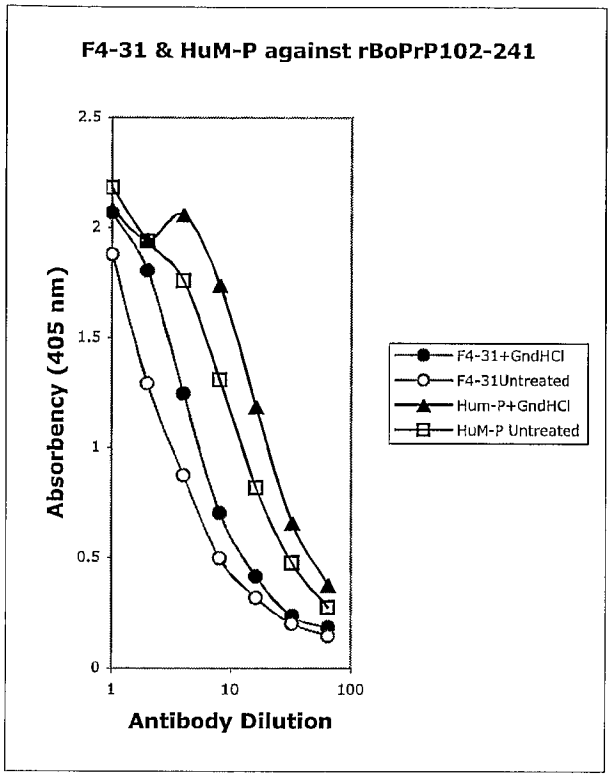
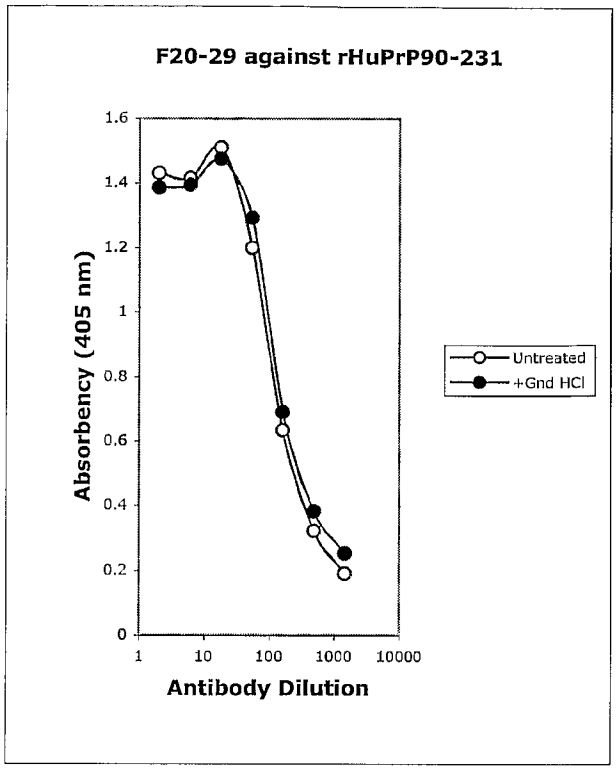
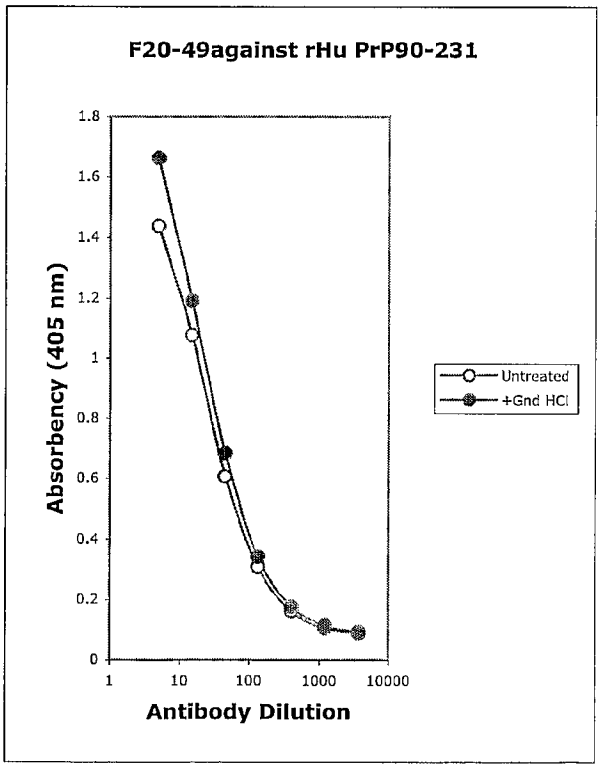



Figure 9A: Effect of GndHCl treatment of the antigen, on subsequent antibody binding in Direct ELISA; F4-31, HuM-P, F20-29, F20-49, and F20-80



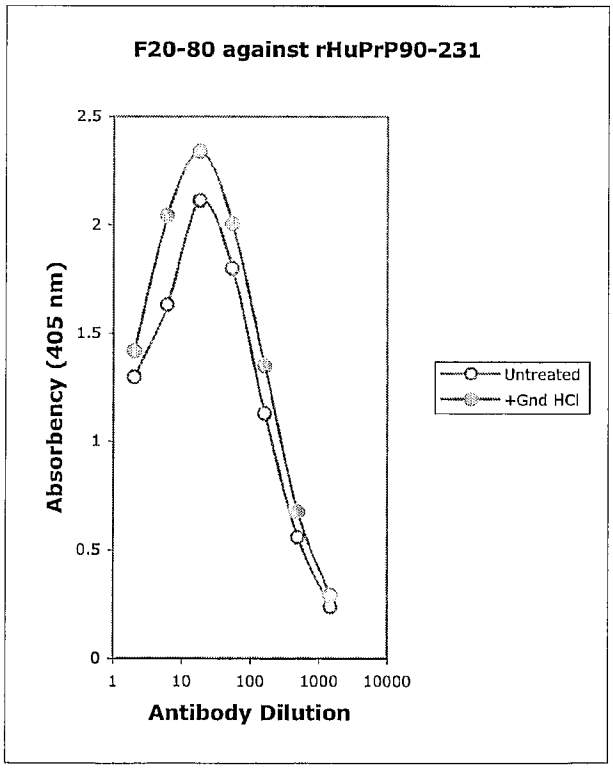
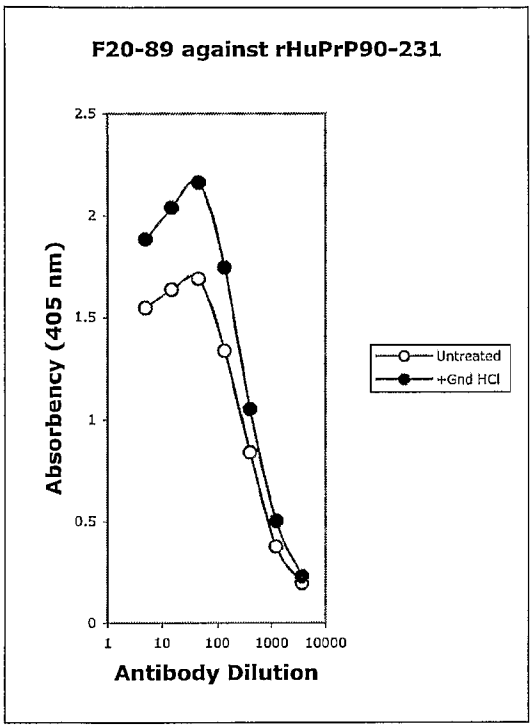
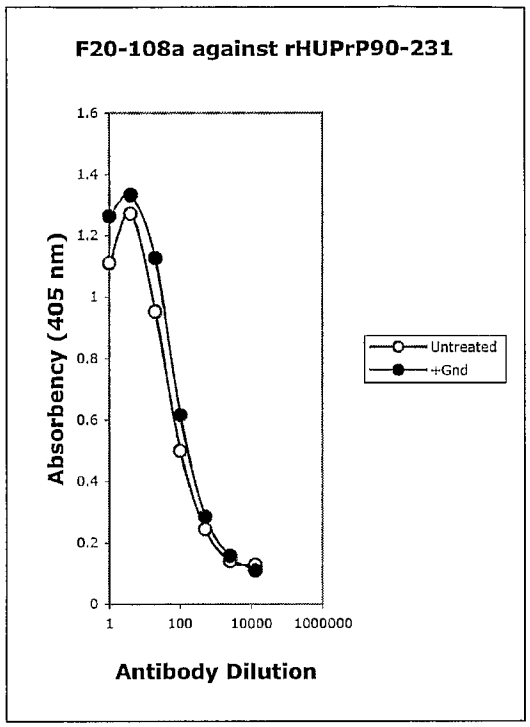



Figure 9B: Effect of GndHCl treatment of the antigen, on subsequent antibody binding in Direct ELISA, F20-89, F20-108a, and F20-130



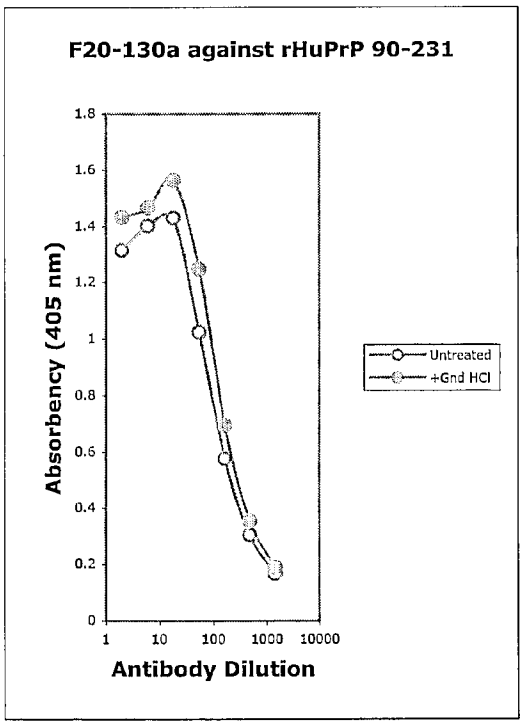



Figure 10 Measurement of infectious prions using a CDI incorporating antibody D-18 versus monoclonal antibody F4-31 as the capture antibody. Samples analyzed were Human, CJD; Guinea Pig, F431; Mouse, RML431; Hamster, Sc237, Sheep, Ov, Deer, CWD and Bovine, BSE infectious brain homogenates.

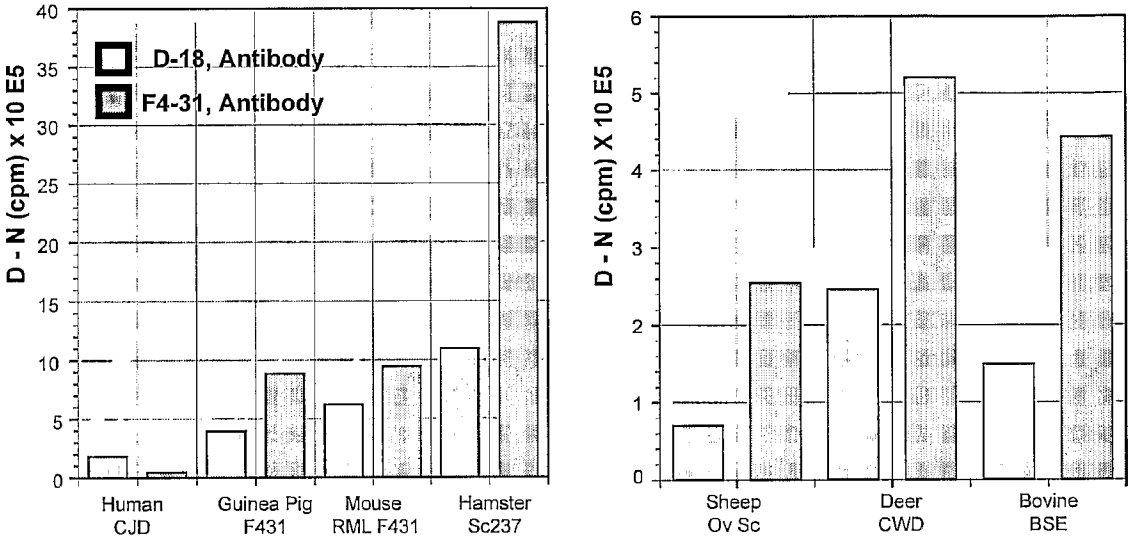


Figure 11 Determination of the capture affinity constant (KD) of mAb F4-31.

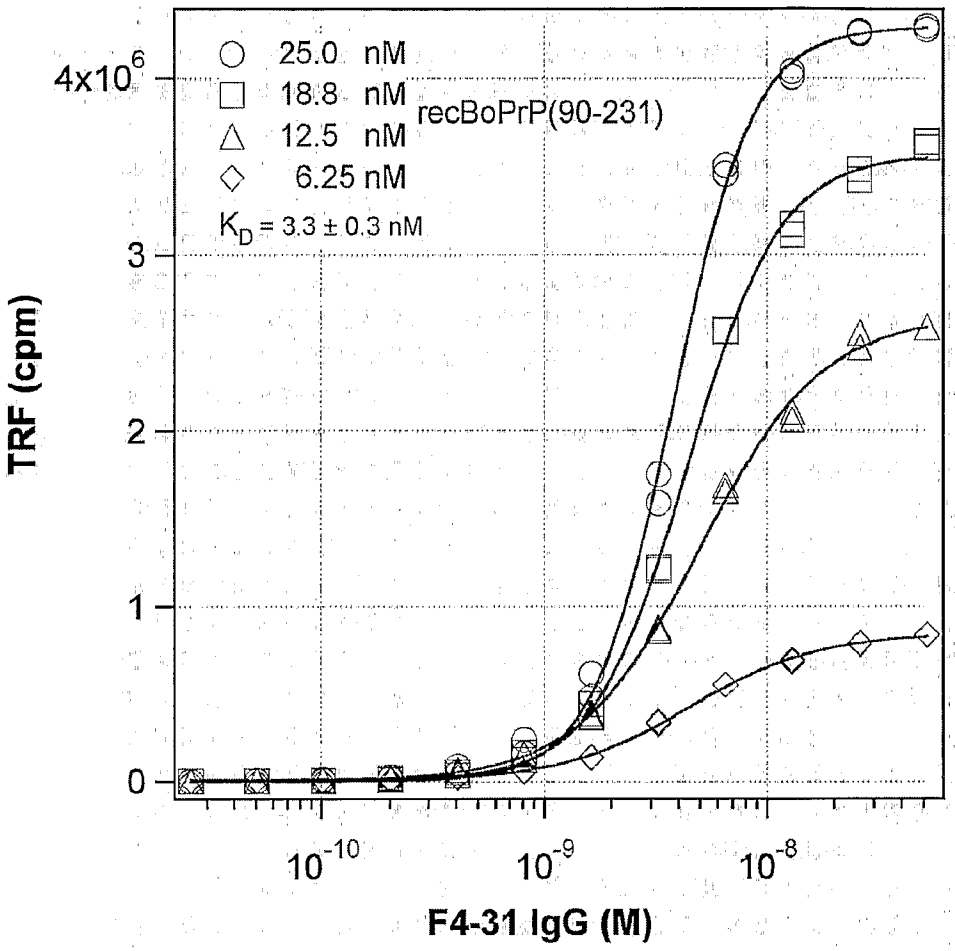
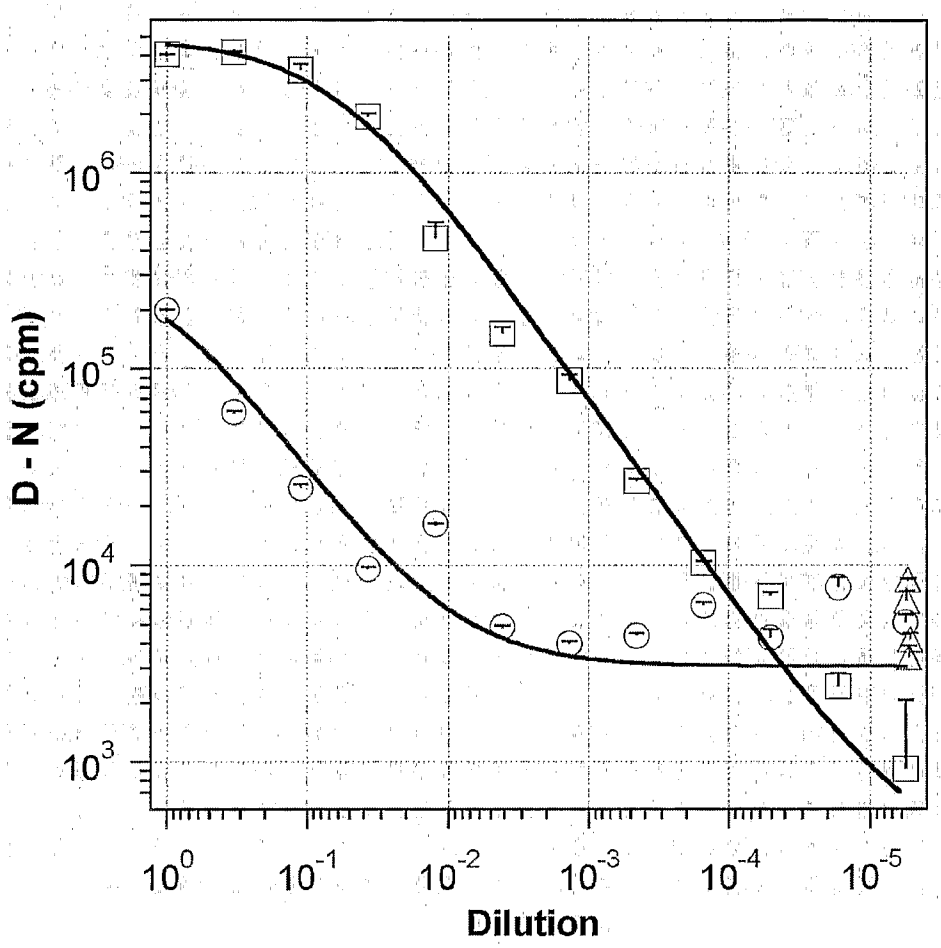


Figure 12 Dilution curve of BSE infected brain homoginate into normal cow brain homogenate. PrPSc was captured in CDI using either D-18 (circle) or F4-31 (square) and then detected by Eu-HuM P. The value D-N is directly porportional to the concentration of PrPSc protein. Data points represent the average of three independent measurements and standard deviation is indicted by bar.



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SAFAR, JIRI G.
STANKER, LARRY

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<210> 34
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<400> 34
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<210> 36
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Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu
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<400> 43
Gly Gln Gly Gly Thr His Gly Gln Trp Asn Lys Pro Ser Lys Pro Lys
1 5 10 15
Thr Asn Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val
20 25 30
Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu
35 40 45
Ile His Phe Ser Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met His
50 55 60
Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn
65 70 75 80
Gln Asn Asn Phe Val His Asp Cys
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<400> 44
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Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys Met Met Glu Arg Val Val
20 25 30
Glu Gln Met Cys Ile Thr Gln Tyr Gln Arg Glu Ser Gln Ala Tyr Tyr
35 40 45
Gln Arg Gly Ala Ser
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<210> 45
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<400> 45
Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr
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Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met
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Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Glu Arg Glu Ser
35 40 45
Gln Ala Tyr Tyr Gln Arg Gly Ser
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<211> 27
<212> PRT
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<400> 46
Phe Val His Asp Cys Val Asn Ile Thr Val Lys Glu His Thr Val Thr
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Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr
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1 5 10 15
Gln Arg Gly Ala
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